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SOME OBSERVATIONS ON THE PATHOGENESIS OF EASTERN EQUINE ENCEPHALOMYELITIS AND LOUPING-ILL IN YOUNG AND OLD ANIMALS, WITH SPECIAL REFERENCE TO ROUTES OF ENTRY OF THE VIRUSES INTO THE NERVOUS SYSTEM.*

By

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INTRODUCTION

Previous observations (Hurst, 1934; Cooke, Hurst and Swan, 1942) have shown that the viruses of pseudorabies and herpes, whether circulating in or injected into the bloodstream of rabbits, do not normally reach the nervous system by this route. From the original focus of infection in skin or muscle, or from an induced visceral focus in the case of virus inoculated intravenously, they travel by the nerves to set up a primary myelitis. Even though introduced directly into the cerebral circulation, the virus of herpes does not readily cause primary encephalitis, unless it be given in conjunction with particles capable of acting as minute emboli and of thus arresting the virus in the cerebral capillaries. The behaviour of these viruses stands in direct contrast to that of infectious myxomatosis of rabbits, which either is distributed uniformly in all parts of the nervous system or, at an early stage of infection, appears first in any part entirely at random (Swan, 1941; Cooke, Hurst and Swan, 1942).

The course of events in some other infections (equine encephalomyelitis, louping-ill), in which during the pre-encephalitic stages virus circulates freely in the blood, is less easily determined. While envisaging the possibility of the virus of equine encephalitis "growing through" the blood-brain barrier, Hurst (1936a) failed in monkeys or in guinea-pigs to obtain definite evidence of its *direct* passage through the barrier. Other considerations suggested the possibility, in some cases, of virus reaching the nasal mucosa from the blood and thence travelling to the brain by the olfactory route, as was believed to occur with poliomyelitis virus injected intravenously into monkeys (Lennette and Hudson, 1935), yellow-fever virus intraperitoneally in hedgehogs and mice (Findlay and Mahaffy, 1936), louping-ill virus intraperitoneally in mice (Burnet and Lush, 1938) and St. Louis virus intraperitoneally in mice (O'Leary, Smith and Reames, 1942). King (1939), however, concluded that in equine encephalomyelitis in the guinea-pig, virus passes directly from blood to brain.

*Much of this work was performed in Australia in 1938–9, before it received the veto of the Commonwealth Department of Health.

In recent years questions of the pathogenesis of the viral encephalitides have been complicated by the recognition of differences in the response to infection of young and of older animals. Theiler (1930) first showed that intraperitoneal injection of neurotropic yellow-fever virus led to nervous involvement only in young mice. Sabin and Olitsky (1937 a, b, c; 1938 a, b, c.), King (1940a), Casals (1940), O'Leary, Smith and Reames (1942) and Lennette and Koprowski (1944) have recorded analogous findings with a number of encephalitogenic viruses. A common state of affairs is that while young and old animals are nearly equally susceptible to virus introduced intracerebrally, as age increases susceptibility to encephalitis following infection by a peripheral route declines sharply. Moreover, in older animals the virus may reach its destination by a route other than that traversed in the immature subject (e.g., Sabin and Olitsky, 1938a).

The experiments described below once more demonstrate the influence of age on the outcome of infection with equine encephalitis and louping-ill and seek to throw further light on the mechanism of nervous involvement.

Experimental

The viruses used in this investigation

In these experiments we have used strains of the virus of Eastern equine encephalomyelitis (EEM) and of louping-ill (LI) identical with those studied previously (Hurst, 1936a, 1931). Both are now removed by 40 to 50 passages from the original host, though the former has long been propagated by intramuscular passage in order to retain its "pantropic" properties.

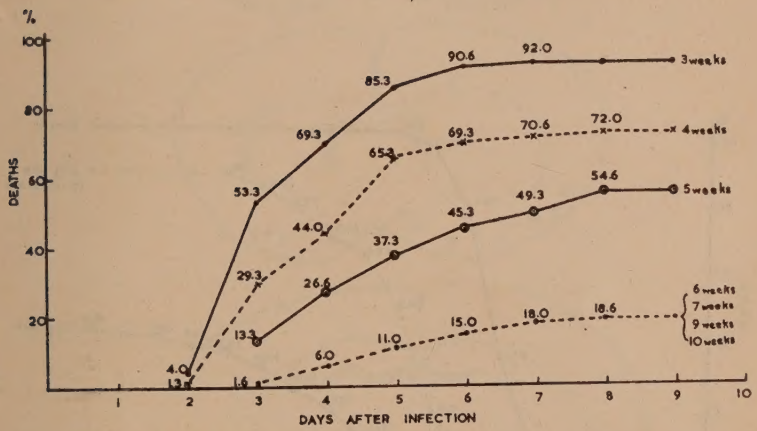
The influence of age of the host on its response to infection

Chart 1 illustrates the results obtained with over 500 mice inoculated intraperitoneally with 10^4 cerebral LD_{50} (per 20 g.) of EEM virus. Whereas most three-week-old animals develop encephalitis and die, under 20 per cent. of those aged six weeks and over do so. Of the total fatalities among three-week-old mice, nearly 60 per cent. occur by the third day whereas among old mice death is delayed until the fifth day or later. Between the third and sixth weeks, susceptibility of the mice to nervous involvement steadily declines without a sharp break in susceptibility at any stage. Other experiments showed that while a dose of 10^7 cerebral LD_{50} in old mice caused an appreciably higher mortality than did one of 10^4 LD_{50}, the deaths did not nearly equal those in young animals.

Inspection of the list of controls in Tables 5 and 6 shows the same phenomenon in guinea-pigs, whereas with rabbits of the ages most used (Table 7) less difference existed between young and old and even recently weaned animals not infrequently escaped fatal cerebral complications.

Chart 2 presents analogous findings in mice inoculated intramuscularly with LI virus, though in this instance even old animals suffer a heavy mortality from peripheral injection of virus.

CHART 1. CUMULATIVE DEATH CURVES OF MICE OF DIFFERENT AGES INOCULATED INTRAPERITONEALLY WITH EASTERN EQUINE ENCEPHALOMYELITIS VIRUS.



Figures from each of three experiments using 25 mice per age-group were substantially the same and were pooled. The curves for the 3rd, 4th and 5th weeks, therefore, represent observations on 75 animals per group. The numbers of deaths per group at 6, 7, 9 and 10 weeks were virtually the same; these observations have been combined into a single curve representing 300 mice. Dose of virus: 10^4 intracerebral LD50.

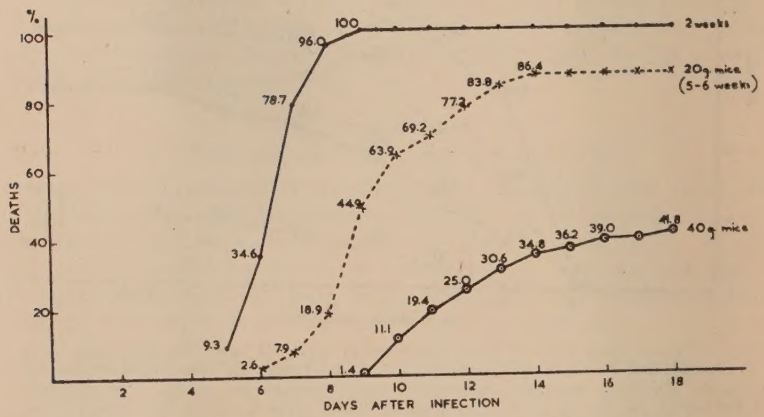
This response of animals of different ages to peripheral inoculation contrasts with their behaviour when the same viruses are inoculated directly into the brain. In two intracerebral titrations of EEM virus using six mice for each ten-fold dilution, the LD_{50} was only one dilution higher in two-week-old than in six-week-old mice. With LI virus the difference was a hundred-fold between three-week-old and six-week-old mice but there was no difference between the latter and old mice weighing over 40g.

Relatively slight though they may be, these differences in the intracerebral titre of the same samples of virus in young and older animals imply that only one-tenth or one-hundredth of the amount of virus necessary in the adult need reach the nervous system of the young animal in order to evoke fatal encephalomyelitis. Were there in addition a much greater proliferation of virus in the extraneural tissues of the young animal, the amount of virus available

to penetrate the nervous system (by whatever route) would be correspondingly great. These two factors combined might go far towards explaining the different responses of young and older animals to peripheral injection of virus.

Hurst (1936a) found that the chief indication of extraneural increase of the virus of EEM lay in the blood-titre. Accordingly, we examined daily the blood of many young and old guinea-pigs for the presence of virus (Table 1). Individual animals manifest

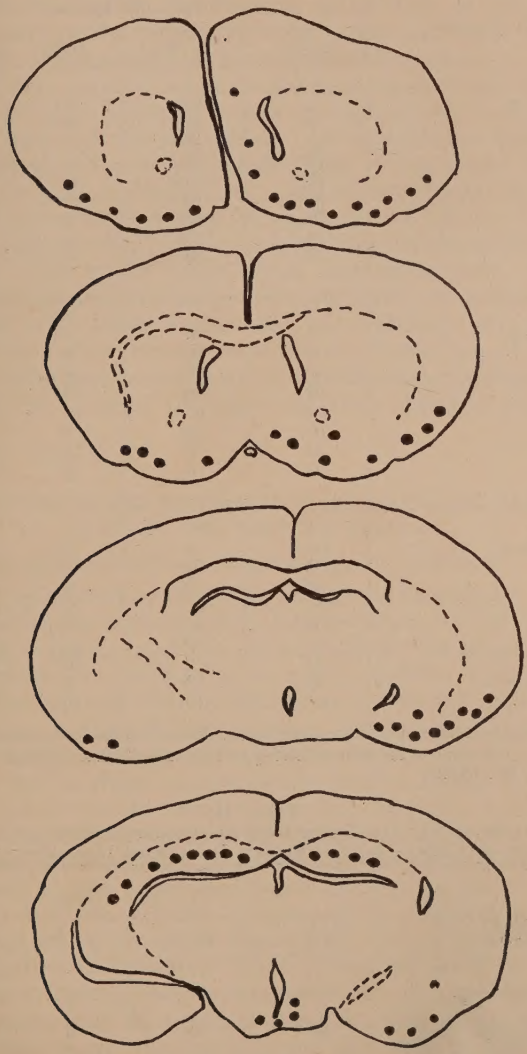
CHART 2. CUMULATIVE DEATH CURVES OF MICE OF DIFFERENT AGES INOCULATED INTRAMUSCULARLY WITH LOUPING-ILL VIRUS.



Pooled figures from three experiments using a total of 75 animals per age-group. Dose of virus : 10^4 intracerebral LD50.

considerable differences in the highest titre reached, so that on occasion the amount of virus detected in the blood of an adult may exceed that in an immature animal. Nevertheless, there can be no doubt that, in general, young animals tend to show higher titres than do mature animals, and that in the young virus is present more consistently over periods of several days. Precisely similar results were obtained in individual young and old mice sacrificed at different intervals after infection. Table 2 shows the titres of pooled samples of blood from young and old guinea-pigs and rabbits; from this it is clear that virus reaches a higher average titre and persists longer in the blood of young guinea-pigs than in that of old guinea-pigs or of young or old rabbits. It is instructive to compare these titres with the mortalities from encephalomyelitis among control animals of the same species listed in Tables 5 to 7.

FIG 1. MOST FREQUENT DISTRIBUTION OF EARLY NERVOUS LESIONS OF EASTERN EQUINE ENCEPHALOMYELITIS IN MICE.



The lesions in 6 early cases were marked on the projected images of the sections. The number of dots in the diagrams indicates the relative severity of involvement of various regions. The concentration of lesions on one side in the upper 3 drawings is due to the fact that in 3 cases lesions were unilateral. In the bottom drawing it is due to the cornu Ammonis having been cut at different levels on the two sides.

TABLE I

DAILY TITRES OF VIRUS OF EQUINE ENCEPHALOMYELITIS IN THE BLOOD OF GUINEA-PIGS INOCULATED INTRAMUSCULARLY.

Weight of animal	*Time after infection*			
	24 hrs.	*48 hrs.*	*72 hrs.*	*96 hrs.*
200—300 g.	0	10^{0}	10^{-3}	10^{-1}
	0	10^{-1}	10^{-2}	10^{-1}
	tr	10^{-2}	tr	DEAD
	10^{-1}	10^{-1}	tr	tr
	10^{-2}	10^{-3}	10^{-1}	DEAD
	10^{-1}	10^{-1}	10^{-1}	DEAD
	10^{0}	10^{0}	0	0
	10^{-2}	10^{0}	0	DEAD
	10^{-1}	10^{-1}	0	DEAD
500—600 g.	0	0	0	0
	0	tr	0	0
	tr	0	0	0
	10^{0}	10^{0}	10^{0}	0
	10^{0}	tr	tr	0
	10^{-1}	10^{0}	0	0
	0	0	10^{-1}	n.t.
	0	0	0	n.t.
700—1000 g.	tr	0	0	0
	0	0	0	0
	10^{0}	10^{-1}	0	0
	tr	tr	0	0
	10^{-2}	tr	tr	tr
	tr	10^{-1}	tr	0
	0	0	10^{0}	tr
	0	0	0	0
	tr	10^{-1}	10^{-1}	tr

tr = only some of the mice receiving undiluted defibrinated blood succumbed.
n.t.= not tested.

TABLE II

DAILY TITRES OF VIRUS OF EQUINE ENCEPHALOMYELITIS IN POOLED SAMPLES OF BLOOD OF GUINEA-PIGS AND RABBITS INOCULATED INTRAMUSCULARLY

Age and species of animal	*Time after infection*			
	24 hrs.	*48 hrs.*	*72 hrs.*	*96 hrs.*
Young guinea-pigs ...	10^{-2}	10^{-1}	10^{0}	All dead
Old guinea-pigs	10^{-1}	10^{0}	0	0
Young rabbits	10^{0}	0	0	0
Old rabbits	tr	0	0	0

We mixed equal amounts of defibrinated blood from each of six animals in a group.
tr = only some of the mice receiving undiluted blood succumbed.

In passing, it may be noted that failure to find virus at any stage in the blood of some adult guinea-pigs does not necessarily indicate that virus spreads by other means. As pointed out previously (Hurst 1936b), the clearing mechanism of the blood is highly efficient so that probably virus is detected only when it is introduced continuously and in considerable amount.

An obvious thought when considering marked age-differences in susceptibility to disease is to seek an explanation in terms of the hormonal status of the individual. In experiments with groups of 30 two-week-old male or female mice, it was found that daily injections for 7 or 10 days of 1 or 2 mg. of testosterone propionate, 0·5 mg. of stilboestrol or 0·25 c.c. of Antuitrin (Parke, Davis and Co.) were without effect on the susceptibility of the animals to the viruses of EEM or LI when given intramuscularly. In other experiments, castration of male or female mice 5 or 30 days prior to inoculation with these viruses did not influence the course of infection. In rather similar experiments, Sabin (1941) failed by inducing precocious sexual maturity to influence the susceptibility of mice to the virus of vesicular stomatitis injected intramuscularly.

THE PRESENCE AND DISTRIBUTION OF VIRUS AND LESIONS WITHIN THE NERVOUS SYSTEM

Equine Encephalomyelitis in Guinea-pigs and Rabbits

Hurst (1936a) showed that after peripheral inoculation the virus of EEM multiplies extraneurally and circulates in the blood. At this time it is absent from the nervous system. In many cases this systemic phase of infection gives place rapidly (young guinea-pigs) or after several days (monkeys) to a nervous phase, during which symptoms of encephalitis appear. Virus is then found principally or wholly in the central nervous system. It is important to know whether in those animals which escape clinical encephalitis the virus penetrates the brain to remain relatively ineffective, or whether it fails altogether to reach the central nervous system. An answer may be suggested by both clinical and experimental observations, as well as by histological studies (see later).

In about half the animals (guinea-pigs and rabbits both young and old) inoculated intramuscularly, body temperature rises within 24 to 30 hours of inoculation. When it does so it tends to be higher in old than in young animals (average rises 1·9° and 1·4°F. respectively). Unless, as in the young guinea-pig, encephalitis supervenes at a very early stage, the temperature then falls, to rise to a new and higher peak soon after virus is detectable in the nervous system (average rises 3·1° and 2·6°F. respectively). In many animals developing encephalitis, only the late rise is seen, and occasionally virus may be found in the nervous system when no pyrexia has yet developed. In animals in which the disease runs its full course with-

out clinical encephalitis, the temperature may rise within the first 24 to 30 hours or it may never rise. In the latter cases it may fluctuate rather more widely than usual around the normal average level or it may remain quite steady; the secondary pyrexia does not develop. When survivors are tested for immunity by intracerebral injection after a lapse of three weeks or more, they may fail to respond or may exhibit a rise of temperature. The pyrexia may or may not be accompanied by nervous symptoms which later regress or may cause death after a delay of several days or as speedily as in a previously uninoculated animal (Table 3). Which of these responses will follow cannot be foretold from the severity of reaction to the earlier intramuscular injection. Animals showing no temperature disturbance whatever and those developing an

TABLE III

RESULTS OF INTRACEREBRAL INOCULATION OF EQUINE ENCEPHALOMYELITIS VIRUS IN RABBITS AND GUINEA-PIGS ESCAPING ENCEPHALITIS AFTER INTRAMUSCULAR INOCULATION.

Age and species of animal	*Response to cerebral inoculation*			
	No reaction	*Pyrexia*	*Pyrexia and non-fatal nervous symptoms*	*Pyrexia, nervous symptoms and death*
Young guinea-pigs ... (under 250 g.)	—	—	—	1
Old guinea-pigs ... ... (over 500 g.)	2	7	—	3
Young rabbits ... ... (700-1000 g.)	3	3	2	1
Old rabbits ... ... (over 2000 g.)	2	5	2	2

These animals were among the controls marked as ' S ' in Tables 5-7.

initial rise of up to 5°F., alike have succumbed in the shortest time to a subsequent intracerebral injection, while similar initial responses have on other occasions been followed by partial or complete cerebral immunity. Morgan, Schlesinger and Olitsky (1942) relate the uncertainty attending intracerebral immunity-tests in EEM to the level of antibody in the blood, which in turn determines that in the interstial fluid of the central nervous tissues.

Table 4 records the findings in animals sacrificed at what were conceived to be appropriate times for demonstration of virus

within the nervous system. The proportion of animals showing no virus is nearly the same as that of animals escaping death in the various groups of controls in Tables 5 to 7. In the great majority of instances where virus was found, the animal had been selected because it showed a secondary rise of temperature suggesting involvement of the nervous system. The negative findings, on the other hand, were in animals taken at a variety of intervals after inoculation because they had no secondary pyrexia, in the hope that they were animals passing through, or destined to pass through, an inapparent infection.

[It should be mentioned that in every experiment at least one young guinea-pig served as a control for the activity of the virus with a batch of old guinea-pigs or of old or young rabbits, and that usually both positive and negative results were associated in one and the same experiment.]

Taking all things into consideration, and particularly the findings in rabbits, it seems probable that most, if not all, animals escaping clinical encephalitis with EEM do so because virus does not penetrate to the nervous tissues and not that they suffer a mild and symptomless infection of the brain. This conclusion is the more credible in view of the great sensitivity of these common

TABLE IV

PRESENCE OF VIRUS OF EQUINE ENCEPHALOMYELITIS IN THE NERVOUS SYSTEM AT DIFFERENT TIMES AFTER INTRAMUSCULAR INOCULATION.

Time	*Young guinea-pigs*	*Old guinea-pigs*	*Young rabbits*	*Old rabbits*
48—72 hours...	9 of 12	4 of 8	0 of 2	0 of 2
73—96 hours...	2 of 2	0 of 3	1 of 5	0 of 3
96—120 hours...	—	3 of 7	1 of 4	0 of 2
121—144 hours...	—	4 of 7	1 of 5	0 of 8
145—192 hours...	—	0 of 3	0 of 2	0 of 12

The table shows the number of positive findings and the total number of brains tested by passage to mice.

laboratory animals to direct intracerebral inoculation of virus; moreover, it is supported by the histological studies reported later. It would not necessarily apply to other animal species or to the same species infected with other viruses. As will be seen presently

it probably does not apply in the case of a few old mice infected with LI.*

A study of the relative times of appearance of a neurotropic virus in different regions of the nervous system may yield information on its route of entry. Using larger numbers of experimental animals than was possible in earlier work, the results shown in Tables 5 to 7 were obtained.

Table 5 shows the distribution of virus in a series of young guinea-pigs killed at an early stage of the nervous infection following intramuscular inoculation in the left leg. The olfactory bulbs and tips of the frontal lobes contained virus in higher concentra-

TABLE V

DISTRIBUTION AND TITRES OF EQUINE ENCEPHALOMYELITIS VIRUS AT AN EARLY STAGE OF THE NERVOUS INFECTION FOLLOWING INTRAMUSCULAR INOCULATION IN GUINEA-PIGS WEIGHING LESS THAN 250 G.

Hours after inoculation	*Virus in*			
	Olfactory bulbs and anterior frontal cortex	*Posterior cerebral cortex*	*Brain-stem*	*Lumbar cord*
66	10^{-1}	10^{-3}	0	0
90	10^{-2}	10^{-5}	0	0
66	10^{-4}	10^{-2}	10^{-4}	0
76	10^{-5}	10^{-4}	10^{-4}	0
66	10^{-5}	10^{-5+}	10^{-3}	0
72	10^{-5+}	10^{-4}	10^{-2}	0
70	10^{-5+}	10^{-5+}	10^{-4}	0
72	10^{-5+}	10^{-5+}	10^{-4}	10^{-1}
66	10^{-6}	10^{-6}	10^{-4+}	10^{-3}
66	10^{-6}	10^{-5}	10^{-1}	10^{-3}
66	10^{-6+}	10^{-4}	10^{-1}	10^{-2}

CONTROLS: One D3; fourteen D4; nineteen D5; three D6; one D8; one D12; two S.

Serial ten-fold dilutions of various parts of the nervous system were each inoculated intracerebrally into 3 mice; + after the titre indicates that an end-point was not reached.

Among the controls, D4 = died on the fourth day, S = survived without showing nervous symptoms.

* With this exception, cerebral infection of mice and guinea-pigs with these strains of virus appears never to be asymptomatic and always to end fatally. In later work, therefore, we have taken survival of the animal as indicating failure of virus to reach the central nervous system. Since also the interval between the development of symptoms and death is relatively constant, the time of the latter event may be accepted as related to the length of the incubation period of the nervous illness.

tion than did the posterior cerebral cortex on five occasions, while in three instances the reverse was true. In one animal the titres of these regions were the same, while in two an end-point was not reached. The brain-stem usually harboured less virus than either of the two preceding areas. The lumbar cord lagged considerably behind other parts of the nervous system, both in the time when virus appeared in it and in the titre of virus attained at a given stage.

TABLE VI

DISTRIBUTION AND TITRES OF EQUINE ENCEPHALOMYELITIS VIRUS AT AN EARLY STAGE OF THE NERVOUS INFECTION FOLLOWING INTRAMUSCULAR INOCULATION IN GUINEA-PIGS WEIGHING 500–860 g.

Hours after inoculation	*Virus in*			
	Olfactory bulbs and anterior frontal cortex	*Posterior cerebral cortex*	*Brain-stem*	*Lumbar cord*
120	0	0	10^{-3}	0
144	0	0	10^{-4}	0
140	0	10^{-1}	10^{-4}	0
66	10^{-2}	10^{-1}	10^{-4+}	0
66	10^{-3}	10^{-1}	0	0
140	10^{-4}	10^{-2}	10^{-4}	0
140	10^{-4}	10^{-3}	10^{-5}	10^{-4}
70	10^{-5+}	10^{-5+}	10^{-1}	0
66	10^{-6}	10^{-6}	0	0
120	10^{-6}	10^{-2}	10^{-1}	10^{-4}
120	10^{-6}	10^{-3}	10^{-2}	0

CONTROLS: Two D4; three D5; seven D6; two D7; one D8; twenty S.

Table 6 presents similar data for old guinea-pigs. The majority of animals showed higher titres in the olfactory bulbs and tips of the frontal lobes than in the posterior cerebrum; only once did the reverse obtain. In several, however, the brain-stem now appeared as a major fount of virus. Again, in the majority of cases, the lumbar cord did not contain virus.

Table 7 gives details of the findings in young rabbits; as far as they go they appear to be similar to those in guinea-pigs. In a series of tests comprising 27 animals (Table 4), virus was not detected in the brains of old rabbits although as can be seen among the controls in Table 7 occasional adult rabbits do develop encephalitis.

Most of these results clearly rule out the possibility of neural spread of the virus from the site of inoculation in the left hind leg, for usually the lumbar cord is involved only in the later stages of infection. They are also difficult to reconcile with simple dissemination by the blood-vessels; as stated previously, in infectious myxomatosis of rabbits, the only indisputably blood-borne infection studied in detail in this respect, virus is distributed wholly at random in the brain *and spinal cord* (Swan, 1941; Cooke, Hurst and Swan, 1942), whereas here the higher regions of the nervous system invariably are first infected. In themselves, however, these results do not appear to warrant final conclusions as to the route followed by virus.

TABLE VII

DISTRIBUTION AND TITRES OF VIRUS OF EQUINE ENCEPHALOMYELITIS AT AN EARLY STAGE OF THE NERVOUS INFECTION FOLLOWING INTRAMUSCULAR INOCULATION IN RABBITS WEIGHING UNDER 1,000 g.

Hours after inoculation	*Virus in*			
	Olfactory bulbs and anterior frontal cortex	*Posterior cerebral cortex*	*Medulla*	*Lumbar cord*
120	0	0	10^{-1}	0
96	10^{-3}	10^{-1}	10^{-4}	0
140	10^{-4}	10^{-4}	10^{-2}	0

CONTROLS : Rabbits under 1,000 g. : One D4 ; one D5 ; one D6 ; fourteen S.
Old rabbits weighing 2,000–2,500 g. : One D5 ; one D 8; nineteen S.
Rabbits just weaned : Seven D3 or 4 ; five S.

Equine Encephalomyelitis and Louping-ill in mice

Owing to the small size of the nervous tissues, and particularly of the spinal cord in mice, no attempt was made to measure the amount of virus present and histological observations were relied on to suggest the point of invasion.

Equine Encephalomyelitis

Mice three-weeks old, mice of 20 g., and old mice weighing not less than 35 g., received virus intramuscularly in the left hind leg and were killed at various stages of infection. Over 200 brains were examined histologically. Many gave negative results because they were taken too early or (particularly in the older age-groups) represented animals which at no time would have shown nervous involvement. Twenty-one brains showing early stages of involvement received detailed attention. The distribution of lesions in these did not differ with the age of the animal.

Prominent among the lesions were the curious foci of disintegration of the ground-substance described by King (1940b). Areas of necrosis of nerve-cells, or necrosis of individual cells, occurred with or independently of this change; either type of destruction might or might not be associated with polymorphonuclear exudate in the tissues or walls of the blood

vessels. In a few places the exudate was not associated with marked destruction of the neurons. On the basis of distribution of these lesions the animals appeared to fall into two groups.

In 7 mice of which only 1 showed very early changes in the olfactory bulbs, lesions occurred in 4 in the diencephalon around the aqueduct, in 2 in the pallium, in 1 in the caudate nuclei, in 1 in the cerebellar cortex and in 1 in the brain-stem. They were of the same type as those in the second group described below; they did not bear an obviously vascular stamp although it cannot readily be supposed that virus reached affected areas by any other route.

In 14 mice lesions were largely or wholly confined to olfactory connections; their characteristic distribution is depicted in Fig 1. Thirteen of the animals showed necrosis in the cortex ventral to the rhinal fissure; in 1 of these the olfactory bulbs were intact, in 1 less affected than the cortex, in 2 more affected than the cortex, and in the remainder about equally affected, In 4 animals lesions were unilateral in the cortex and the corresponding olfactory bulb, in 1 unilateral in the cortex with both bulbs involved, and in 2 very unequal on the two sides with similar inequality in the olfactory bulbs. In 1 animal, only the olfactory bulbs suffered early changes. The areas of necrosis occurred more frequently in the inner layers (molecular, mitral and inner granular layers) of the bulbs than in the two outer layers. In all these animals the pallium was wholly free from lesions, except perhaps for the part immediately adjacent to the rhinencephalic cortex. In 7 bilateral lesions were present in the cornu Ammonis, in 2 near the ventral part of the infundibulum, in 1 ventral to the anterior commisure, in 1 in the septum pellucidum, and in 2 in the diencephalon around the aqueduct. The brain-stem, cerebellum and spinal cord were free from abnormality.

In 3 mice at a more advanced stage of infection lesions in the spinal cord were more severe than those at higher levels; in 18 spinal lesions were much less severe or absent.

In none of 20 mice escaping nervous symptoms until killed on the 18th day were lesions detected in the central nervous system. This finding reinforces the suggestion made previously that subclinical infection of the nervous system in EEM does not occur in the animal species considered in this paper.

To summarise, histological studies on EEM in the mouse agree with experimental studies in guinea-pigs and rabbits in showing that virus is not distributed in the nervous system wholly at random, as might be expected if it were carried by the blood-stream; the spinal cord is involved only at a relatively late stage (since virus was given intramuscularly in the hind leg this fact excludes neural spread of virus) and the earliest changes appear at higher levels of the neural axis. Nevertheless, in about one-third of the animals the situation of early lesions in the cerebrum can be reconciled only with a distribution of virus by the blood vessels even though no clear histological evidence indicating their participation exists. In the other two-thirds of the animals in which the olfactory connections bear almost the whole brunt of the attack the lesions are exactly similar in type and we must assume either that the vessels in these areas are more easily passed by the virus or that the nervous

tissues here are more susceptible to the virus, or that virus may travel by an alternative route, as for instance by way of the nasal mucosa to the olfactory bulbs. The occasional absence of lesions in the olfactory bulbs when other olfactory areas are already affected and the relatively infrequent preponderance of lesions in the bulbs, cast doubt on the last assumption; on the other hand, the not infrequent unilateral distribution of moderately advanced and widespread lesions appears difficult to explain on either of the first two hypotheses.

Louping-ill

Table 8 shows the distribution of lesions in the brains of mice inoculated intramuscularly in the left leg with the virus of LI.

TABLE VIII

DISTRIBUTION OF LESIONS OF LOUPING-ILL IN THE NERVOUS SYSTEMS OF MICE INOCULATED INTRAMUSCULARLY IN THE LEFT LEG.

Mouse No.	*Olfactory bulbs*	*Cerebral hemispheres*	*Brain-stem and cerebellum*	*Spinal cord*
		Two-week-old mice		
34	+	++	±	0
35	++	±	++	+
36	±	±	±	0
37	+	±	±	0
38	±	±	±	0
39	0	±	±	±
45	++	+	++	++
46	±	±	±	0
		20 g. mice		
49	0	+	++	0
51	+++	+++	±	0
52	++	+	0	0
56	+++	+	0	0
57	++	±	0	0
58	0	±	+	0
66	+++	++	+	0
68	+++	+++	+	+
69	+++	+++	+	0
72	++	+	0	0
		Mice over 35 g.		
75	0	++	++	++
81	0	++	++	0
83	+++	++	±	±
84	+++	++	±	0
87	+	++	+	0
88	+	++	±	0
92	++	0	0	0
21	+++	++	±	0
23	+++	++	0	0
24	++	+	0	0
25	++	+	±	0
27	+++	±	0	0
28	++	++	+	+

Two types of lesion were present in these cases. One type was essentially vascular and perivascular. Some vessels were cuffed with mononuclear cells, and the smaller ones affected showed thickening of the endothelium and of the adventitia. Around the vessel microglial cells were proliferating, and scattered nerve-cells had often undergone acute necrosis (cf. Hurst, 1931, figs. 2 and 3). The second type of lesion consisted in massive necrosis of neurons, sometimes associated with vascular and perivascular changes but at other times existing quite independently. The latter lesions occurred usually in the olfactory bulbs, the cortex ventral to the rhinal fissure, the cornu Ammonis or the Purkinje layer of the cerebellum. The perivascular lesions occurred in the olfactory bulbs, etc., but more commonly in the general cortex, basal ganglia, brain-stem and spinal cord (when affected).

In general, as will be seen from Table 8, the anterior parts of the nervous system were more constantly affected than more caudal regions; as in EEM the spinal cord was usually affected only when severe lesions existed at a higher level. In but few animals were lesions absent from the olfactory bulbs when present elsewhere; often in the oldest animals the changes here exceeded in intensity those in other parts.

Among the two-week-old animals, Nos. 34, 38 and 45 showed pure neuronal lesions in the olfactory bulbs (in 34 and 45 limited to the innermost layer of granules and the adjacent pyramidal cells), 34 in many parts of the cortex and basal nuclei, 35 and 45 among the Purkinje cells of the cerebellum, and 45 in the anterior and posterior horns of the spinal cord. In other affected parts of the nervous system of these animals and in other mice of the group, vascular and perivascular lesions were prominent and neuronal necrosis related to these. Contrasted with the other groups, the two-week-old mice exhibited more uniform distribution and severity of lesions throughout the brain.

Among the 20 g. animals, Nos. 49 and 58 presented no lesions in the olfactory bulbs. In 49 and 57 lesions were solely of the vascular and perivascular type. In 56, 68 and 69 the olfactory bulbs showed purely neuronal lesions while the cerebrum was the seat of vascular and perivascular changes. Nos. 51 and 58 exhibited almost pure neuronal lesions, respectively of widespread distribution and confined to the cerebellum. brain-stem and diencephalon. Nos. 52, 66 and 72 showed unilateral neuronal lesions in an olfactory bulb and less advanced unilateral necrosis in the rhinencephalic cortex, with or without perivascular lesions elsewhere. In the olfactory bulbs of all animals of this group lesions when present occurred in both inner and outer layers.

Of the old mice Nos. 75 and 81 showed purely vascular and perivascular lesions without involvement of the olfactory bulbs, and 28 similar changes with focal vascular and neuronal lesions in the bulbs. No. 87 showed massive necrosis in the cornu Ammonis, together with lesser changes in the rhinencephalic cortex and olfactory bulbs and mild vascular and perivascular changes elsewhere. Nos. 88, 24 and 27 showed more or less neuronal damage in the bulbs with vascular and perivascular lesions in parts of the brain other than the olfactory areas. Nos. 83, 84 and 25 exhibited moderate or severe damage to the olfactory bulbs, primary neuronal necrosis in the rhinencephalic cortex, and mild or moderate vascular and perivascular changes elsewhere. In Nos. 21 and 23 the neuronal lesions in the olfactory bulbs were unilateral and were associated with less severe unilateral lesions in the rhinencephalic cortex;

vascular changes were wanting. In No. 92 we made a further interesting observation. One olfactory bulb was severely damaged, and from the type of cellular reaction it was evident that the lesions were of several days' standing. No lesions existed in other parts of the nervous system, suggesting that in this case the lesions in the bulb represented an attack on the nervous structures arrested at this point and destined to progress no further. In most instances in this series all layers of the olfactory bulbs were the seat of changes, but in No. 25 lesions were located largely in the outer layers.

Thus LI differs from EEM in mice in that there tend to be differences in the histological picture according to the age of the animal. In young mice lesions are more often fairly uniformly distributed throughout the cerebrum than is the case in older animals. While individual lesions may be vascular and perivascular or purely neuronal in type, in animals of this age-group the latter is never the only type present; on the other hand, vascular and perivascular lesions alone may be found. In older animals either type of lesion may be present alone, or both may be associated; there is a greater tendency for severe neuronal necrosis to be localised predominantly in olfactory areas (where it is often more advanced anteriorly) with or without vascular and perivascular changes elsewhere. Such lesions may be wholly or mainly unilateral, or even confined to and apparently arrested in one olfactory bulb, suggesting the possibility of a subclinical involvement of the nervous system not observed in EEM. As in EEM the spinal cord is usually affected only when severe lesions occur at a higher level.

THE EFFECT OF REMOVAL OF THE OLFACTORY BULBS PRIOR TO INFECTION

Hurst (1936a) found that removal of the olfactory bulbs from guinea-pigs did not prevent cerebral infection with EEM virus given intramuscularly, as might be expected if the virus travelled *via* the nasal mucosa and olfactory connections. The number of animals used was, however, too small to have statistical significance were the olfactory route concerned in only a proportion of the animals. The experiments recorded in Table 9 involved 150 mice from which the bulbs had been removed three weeks prior to intramuscular or intracerebral inoculation of the viruses EEM or LI. They show that the operation did not prevent infection of the nervous system after intramuscular injection of virus; in fact, with one dilution of each virus there resulted a significant (LI) or almost significant (EEM) heightening of susceptibility. In EEM the average incubation period appeared to be increased as compared with that in controls. It is important to note that susceptibility to direct intracerebral inoculation of a limiting dilution of EEM was heightened to an even greater extent; suggesting a lasting effect of the surgical operation and the unsuitability of animals thus treated for experiments designed to elucidate the route of infection in the intact animal.

TABLE IX

RESULTS OF INOCULATING EQUINE ENCEPHALOMYELITIS OR LOUPING-ILL VIRUS INTO MICE THREE WEEKS AFTER REMOVAL OF THE OLFACTORY BULBS.

Virus	*Dilution*	*Route of inoculation*	*Mortalities in groups of 30 mice*			
			Surgically treated animals		*Controls*	
			Deaths	*Mean period of survival after virus (in days)*	*Deaths*	*Mean period of survival after virus (in days)*
EEM	10^{-3}	Intramuscular	14	9·6	7	7·9
	10^{-4}	,,	4	11·8	4	7·8
	10^{-7}	Intracerebral	11		1	
LI	10^{-4}	Intramuscular	18	13·6	21	12·8
	10^{-5}	,,	16	15·8	3	18·3

At the time of inoculation these mice weighed approximately 20 g.; the controls, which had gained weight more rapidly than those from which the olfactory bulbs had been removed, were the heavier.

ATTEMPTS TO FACILITATE THE DEVELOPMENT OF ENCEPHALITIS IN MICE INOCULATED INTRAMUSCULARLY

If the viruses under consideration are ordinarily carried to the brain by the blood-stream, and if their transit across the blood-brain barrier is the simple and direct passage implied in the writings of some authors, then it should be possible to increase the proportion of animals developing encephalitis by measures which increase the permeability of the barrier. Alternatively, one might by such measures substitute direct blood-to-brain passage of the virus for some more devious route of infection, with consequent alteration of the time needed for the appearance of symptoms.

Hurst and Davies (1950) have recently considered at length the effect of various drugs and procedures reputed to favour the passage of other substances across the blood-brain barrier. Using 15 dyes, 3 convulsant drugs, morphine, sulphanilamide and sulphanilic acid as the test-substances, they found no drug or treatment having a consistent effect on all. Their findings recalled the conclusions of Stern, Kassil and Lokschina (1927), that measures increasing the penetration into the brain of one substance do not necessarily increase penetration of other substances more or less similar chemically or physico-chemically. The experiments therefore gave no lead as to which procedure would be most likely to be

TABLE X

EFFECT OF VARIOUS TREATMENTS APPLIED WHEN VIRUS IS CIRCULATING IN THE BLOOD-STREAM ON THE MORTALITY RESULTING IN MICE FROM INTRAMUSCULAR INOCULATION OF THE VIRUS OF EQUINE ENCEPHALOMYELITIS OR LOUPING-ILL.

Treatment	*Mean weight of mice*	*Mortalities in groups of 25 mice*							
		Equine encephalomyelitis				*Louping-ill*			
		Treated Group		*Controls*		*Treated Group*		*Controls*	
		Deaths	*Mean period of survival (in days)*	*Deaths*	*Mean period of survival (in days)*	*Deaths*	*Mean period of survival (in days)*	*Deaths*	*Mean period of survival (in days)*
Intramuscular injection of 0·25 or 0·4 cc. 50 per cent. glycerol (v./v.) according to weight of animal.	20 g.	20	4·6	22	5·0	16	9·4	7	12·3
	,,	20	4·9	24	5·2	17	9·7	10	9·7
	,,					15	9·5	20	9·5
	43 g.	12	6·0	7	6·7	13	11·4	12	11·8
	,,	9	9·1	7	7·3	6	11·0	9	10·3
Intravenous injection of 0·6 or 1·0 unit of insulin, according to weight of animal, to mice starved for 18 hours	20 g.	17	4·9	19	5·0	12	10·2	6	9·2
	,,	22	5·3	23	4·9	21	9·8	14	12·2
	,,					23	9·0	21	9·4
	39 g.	5	10·6	3	6·0	12	12·2	10	11·9
	,,	9	8·9	6	7·0	12	11·4	5	11·8
Exposure to coal-gas to produce unconsciousness for a total of 10 minutes	20 g.	15	4·9	16	4·9	15	8·7	10	9·7
	,,	22	5·2	22	4·8	25	9·8	20	9·4
	42 g.	6	7·6	7	6·7	15	10·5	12	11·8
	,,	8	6·7	7	7·3				

Intravenous injection of 0·005 mg. adrenalin and 0·1 unit pituitrin in 0·1 cc. water/20 g.	20 g.	21	4·7	24	3·5	14	11·4		
	,,	13	4·5	17	5·3				
	,,	8	5·0	11	5·2				
	40 g.	16	4·3	5	6·6				
	,,	16	4·2	19	5·8				
	,,	10	4·6	6	6·0				
Intravenous injection of 20 mg. urethane in 0·1 cc. water/20 g. ...	20 g.	8	6·9	17	5·6	17	10·9		
	,,	19		19					
	,,	21		21					
	40 g.	3	7·8	8	8·6			23	9·7
Intravenous injection of 16 mg. sodium lactate in 0·1 cc. water/20 g. ...	20 g.	20		13		20	10·4		
	,,	12	6·0	11	5·6				
	,,	12		12					
	40 g.	8	6·2	11	8·6				
Intravenous injection of 5 mg. theocin in 0·1 cc. water/20 g.	20 g.	13	6·0	17	5·6	17	10·9		
	,,	19		23					
	,,	13		13					
	,,	7		13					
	40 g.	4	10·8	11	8·6	7	12·7		
Intravenous injection of 8 mg. histamine in 0·1 cc. water/20 g. ...	20 g.	21		20				10	12·1
	,,	16		13					
	40 g.					9	12·0		
Intravenous injection of 50 mg. hexamine in 0·1 cc. water/20 g. ...	20 g.	20	5·1	17	4·9	14	11·2	12	11·4

effective in the case of viruses, and many of the measures examined by Hurst and Davies were employed against both EEM and LI. We administered the drug or treatment 24 and again 48 hours after intramuscular injection of EEM virus into groups each of 25 mice, and 24, 48 and 72 hours after LI, that is to say at times when virus is known to be circulating in the blood (Table I). Table 10 presents the results.

Glycerol. King (1942) found that *sudden* dehydration resulting from injection of 50 per cent. glycerol greatly facilitated invasion of the brain by EEM virus. After the same treatment, Hurst and Davies observed an increased passage into the brain of sulphanilamide, sulphanilic acid and many dyes, and an increased mortality from strychnine and picrotoxin; the action of morphine and that of cocaine were decreased. In the present experiments (Table 10) no significant results were obtained in young adult or old mice infected with EEM. With LI in young adult mice an apparent effect in two experiments was offset by the reverse in a third; again the mortality in old mice remained unaffected. These results were so much at variance with those of King (who administered virus and glycerol at the same time) that we then repeated his experiments exactly. In three tests with EEM and two with LI we failed completely to influence mortality from encephalitis by giving a dose (0·25 c.c./20 g.) of 50 per cent. glycerol sufficient in itself to kill from the immediate consequences about one-third of the mice. In our hands, therefore, this treatment, which undoubtedly increases the passage into the brain of many chemical substances, did not facilitate entry of the viruses of EEM or LI.

Insulin. By insulin-shock, Findlay (1942) localised in the brains of adult mice neurotropic yellow-fever virus injected intraperitoneally; normally, only very young mice develop encephalomyelitis following this route of inoculation (Theiler, 1930). In EEM the only effect was a lengthening of the mean period of survival in older animals, an effect which appeared to be significant. In LI, however, the treatment tended to increase mortality, and in the aggregate the results appeared significant.

Coal-gas. By exposing mice to coal-gas, Findlay (1942) obtained results similar to those with insulin. In our experiments also the results were very similar; we observed no effect on mortality from EEM and a significantly increased mortality from LI. In other experiments (Hurst and Davies) exposure to coal-gas increased the passage into the brain of several dyes and of sulphanilic acid, while the entry of sulphanilamide was decreased.

Adrenalin and pituitrin. Friedemann (1937) used adrenalin for its "auxo-neurotropic" action and combined it with pituitrin to minimise undesirable side-effects. According to him, when given intravenously it increases the permeability of the blood-brain barrier for substances which normally pass through. Hurst and Davies found that it increased the passage of some dyes and the

mortality from convulsant drugs; it did not increase the amount of sulphanilamide or sulphanilic acid passing into the brain and only slightly the analgesic effect of morphine. In the experiments detailed in Table 10 it tended to diminish mortality in young animals (EEM and LI) and to increase it in older animals (EEM); the effects for old animals approached significance. Moreover, the mean period of survival in older animals was significantly lowered.

Theocin. Claimed by Fröhlich and Zak (1927) and Franceschetti and Wieland (1928) to increase the permeability of the blood-brain and blood-cerebrospinal fluid barriers, this substance has no apparent effect on the passage of sulphanilamide or sulphanilic acid, decreases the effect of morphine, and increases that of convulsant drugs (Hurst and Davies). Its use in animals infected with EEM or LI resulted in a tendency towards diminished mortality, with a significantly increased mean period of survival in old animals.

Urethane, sodium lactate, histamine and hexamine were without clear-cut or consistent action. The reasons for their use and their effect on the pentration into the brain of chemical substances can be found in the paper of Hurst and Davies.

Summarising these experiments, it appears that the use of of insulin or of coal-gas at a time when virus is circulating in the blood-stream, leads to increased mortality from LI but not from EEM. Findlay explains the corresponding effect with neurotropic yellow-fever virus as due to seepage of virus through the walls of vessels damaged by anoxia. Adrenalin-and-pituitrin possibly increased mortality among old animals infected with EEM and certainly diminished the mean period of survival. The intramuscular inoculation of glycerol, although appearing on some occasions to increase mortality in young animals infected with LI, was not consistent in its effect, and under the exact conditions of King's experiments we were unable to demonstrate an effect with either EEM or LI. No other treatment facilitated the development of encephalitis due to either virus.

DISCUSSION

The experiments described in this paper confirm for EEM and demonstrate for LI the striking difference existing between young and old animals in their susceptibility to encephalitis following intramuscular injection of virus. We believe that this difference may well be accounted for largely on a quantitative basis. To direct intracerebral inoculation of EEM virus, the young mouse is about ten times more susceptible than the adult, and to LI virus one-hundred times more susceptible. In young animals, on an average, EEM virus multiplies much more abundantly and circulates in the blood longer. It is not unreasonable to suppose that these are important determining factors of the much higher mortality among the young. O'Leary, Smith and Reames (1942) have advanced a

rather similar explanation of their findings with St. Louis virus. Neither Sabin (1941) nor we have obtained evidence that hormonal factors contribute to the difference in susceptibility. Finally, the observations on EEM suggest that guinea-pigs, rabbits or mice escaping clinical encephalitis do so because the virus has not invaded the central nervous system, and not because they suffer an inapparent cerebral infection; with LI in old mice, on the other hand, the histological findings point to occasional subclinical infection of a part of the central nervous system (olfactory bulbs).

The present and earlier work establishes clearly that the viruses of Eastern EEM and LI do not commonly reach the central nervous system by way of the peripheral nerves from an intramuscular site of inoculation. At an early stage of nervous involvement, virus and/or lesions are nearly always absent from the spinal cord when higher regions of the neural axis already show both. In the case of viruses known to circulate in the blood, the obvious tendency when a neural mechanism is exonerated from conveying virus to the central nervous system is to seek evidence of a vascular distribution. The behaviour of some viruses leaves little doubt that they are so distributed. Thus the virus of infectious myxomatosis of rabbits is distributed uniformly in all parts of the central nervous system or, at an early stage of infection appears first in any part entirely at random Swan (1941); Cooke, Hurst and Swan (1942). Again, the histological evidence leaves little doubt that the virus of hog-cholera is spread by the blood, for lesions are scattered more or less evenly over the grey and white matter of brain and spinal cord and the vascular apparatus is visibly and heavily involved (Seifried, 1931, and personal observations).

The strain of EEM used in this work does not furnish equally convincing evidence of its method of spread. The notable absence of virus and lesions from the spinal cord until a relatively late stage of infection, the histological characters of the lesions in many mice, the frequent restriction of early lesions to the olfactory areas and the not infreqent unilateral distribution of these lesions are difficult to reconcile with the random distribution expected of an agent disseminated by the blood-stream. To achieve a reconciliation we should have to assume not only a selective penetrability of the vessels of the cerebrum as opposed to those of the cord, but a selective penetrability of the vessels of a particular functional area, not uncommonly on one side only. An alternative assumption, that the nervous tissues in the affected areas are innately more susceptible to virus, appears equally unable to explain the unilateral occurrence of lesions. Yet the more detailed study of a larger number of animals than was possible on an earlier occasion Hurst (1936a) has not produced conclusive evidence supporting the further possibility then suggested, that virus may reach the brain by way of the olfactory nerves from the nasal mucosa. In one of 13 mice exhibiting moderately advanced changes in the rhinencephalic cortex, the

olfactory bulbs were normal and in a second were less affected than the central areas; in only three were lesions in the bulbs more severe than in the cortex or present in the bulbs when absent from the cortex. Moreover, in about one-third of the animals examined, the earliest lesions (exactly similar in type to those in the olfactory areas of other mice) appeared in situations which could hardly be reached by any route other than the vascular. It may perhaps be recalled that in our former work we did not succeed in isolating the Eastern EEM virus from the nasal washings of infected animals, though we were successful in similar attempts with the Western virus. The present study leaves it doubtful, therefore, whether the Eastern EEM virus reaches the brain by way of the nasal mucosa.

The case of LI in mice is rather different. Obviously vascular and perivascular lesions and purely neuronal lesions may occur together (but usually in different situations) in a given case, or either may be present without the other; young and older animals differ in the relative frequency with which they show the two types of change. Among older animals one more frequently encounters cases in which there is a definite gradient in the severity of the lesions from the olfactory bulbs centrally, and even occasional cases in which lesions of some standing have apparently been arrested in the olfactory bulbs. This kind of evidence, agreeing with that obtained by Lennette and Hudson (1935), Burnet and Lush (1938) and O'Leary, Smith and Reames (1942), points to the olfactory route of infection as an alternative to the vascular. In many animals, no doubt, virus tends to go by either route, and the exact outcome depends more or less on chance. In older animals, perhaps, the olfactory mechanism is operative more frequently than in the young, in which vascular phenomena are more widespread and of more constant occurrence. There is, however, no indication that the mode of infection is absolutely different at the various ages, as it appears to be with some viruses (Sabin and Olitsky, 1938a).

This being so, it is not surprising that removal of the olfactory bulbs does not render mice refractory to cerebral infection with EEM or LI. On the contrary, animals thus treated appear to be more susceptible to encephalitis. We must emphasize that the enhanced susceptibility extends also to direct intracerebral infection; evidently the operative procedure makes the brain more vulnerable, and with the viruses under consideration the technique is of little value for establishing the course of events in the intact animal.

In considering the probability that some viruses may pass the blood-brain barrier, few authors have given attention to the precise mechanism involved. In current physiological literature the term "permeability" as applied to a biological membrane refers to the

ease or otherwise with which an ion or a smaller or larger molecule passes the membrane. To speak of permeability of the blood-brain barrier to viruses suggests that penetration of the defences of the nervous system is a process analogous to that of diffusion. From what we know of the size and complexity of viruses, and of the difficulty experienced in passing the barrier by similarly charged particles of much smaller size, it would seem that some other mechanism must be concerned. Hurst (1936a) could not demonstrate direct passage of the virus of EEM from blood to cerebrospinal fluid or brain, and considered the possibility of a "growth through" the barrier. Observations with herpes (Cooke, Hurst and Swan, 1942) showed that this virus at any rate does not easily infect the brain even when injected directly into the cerebral circulation; the technique used in the experiments was such as to avoid the criticism subsequently levelled by Friedemann (1943) at similar experiments by Doerr and Hallauer (1936), who reached an opposite conclusion.

It seemed that evidence on this point might accrue if we adopted measures reputed to increase the amount of one or other chemical substance passing from blood to brain (Hurst and Davies, 1950). We applied these measures at times when virus is known to circulate in the blood; an increased rate of mortality would have suggested that passage of virus across the barrier is a process on all fours with the penetration of a dye or other solute. Administration of insulin or of coal-gas to animals incubating LI caused the death-rate to rise, but in EEM these procedures were without effect; in any case, the treatment might be expected appreciably to damage the walls of the blood-vessels. Adrenalin-and-pituitrin probably increased mortality and certainly expedited the development of encephalitis in old (but not in young) mice infected with EEM, but it is difficult to imagine that this is a simple case of increased permeability, in the true sense of the term. Glycerol, which King (1942) had found to facilitate cerebral infection with EEM, failed in our hands to influence the course of this disease and gave only doubtful results with LI; in an exact repetition of King's experiments we could demonstrate an effect on neither infection. Yet glycerol is most potent in favouring the transfer of many chemical substances from blood to brain (Hurst and Davies). The other measures employed yielded negative results or had the opposite effect to that intended. These findings suggest that, whatever the mechanism by which viruses pass from blood to brain, it is quite different from that allowing the passage of dyes, and so on; the term permeability should not, therefore, be used in this connection.

SUMMARY

Young animals infected intramuscularly with Eastern equine encephalomyelitis or louping-ill develop encephalitis sooner and

die in far larger numbers than do older animals. Thus with equine encephalomyelitis 92 per cent. of three-week-old mice died by the 7th day, and only 18·6 per cent. of adult mice by the 8th day. With louping-ill 100 per cent. of two-week-old mice died by the 9th day, and only 41·8 per cent. of old mice by the 18th day. The transition between the greater susceptibility of young animals and the lower susceptibility of old animals is gradual.

The difference in response of young and old animals can probably be attributed to a greater susceptibility of the cerebral tissues (ten-fold in the case of equine encephalomyelitis, a hundred-fold in the case of louping-ill), combined with the fact that virus multiplies more and circulates longer in the blood of the young. There is no evidence that hormonal factors play a part.

With these two viruses no absolute difference exists in the route by which the nervous system is invaded in young and old animals. Neither virus commonly spreads by way of the peripheral nerves. There remain as alternatives (*i*) spread by the blood-stream, and (*ii*) infection by way of the nasal mucosa and olfactory apparatus. The difficulty in reaching a decision as to the route followed in the case of equine encephalomyelitis is discussed; no conclusive evidence in favour of the second alternative has been adduced. In louping-ill, on the other hand, the evidence suggests that either or both routes may be used according to circumstances. In louping-ill in old mice the nervous infection may be localised and arrested in the olfactory bulbs.

Removal of the olfactory bulbs does not prevent infection of the nervous system with virus given intramuscularly; on the contrary it may enhance susceptibility both to intramuscularly and to intracerebrally injected virus.

Some measures reputed to increase the passage of chemical substances from blood to brain do not alter the incidence of nervous involvement in equine encephalomyelitis or louping-ill. The development of encephalomyelitis in louping-ill is favoured when, during the period in which virus circulates in the blood, the animals are exposed to coal-gas or subjected to insulin-shock. These measures have no effect in equine encephalomyelitis, in which disease, however, the intravenous administration of adrenalin-and-pituitrin tends to increase the number of cases of encephalitis and expedite their appearance in old animals only. It is probably incorrect to use the term "permeability" in relation to the passage of viruses from blood to brain; the transit is here regarded as a "growth through" the wall of the vessel.

When a mouse, guinea-pig or rabbit escapes encephalitis after peripheral inoculation of the virus of equine encephalomyelitis, it does so because virus has not reached the nervous system and not because it has suffered an inapparent nervous infection. In louping-ill cases of subclinical involvement of the nervous system are seen occasionally.

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SCRAPIE IN SHEEP

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Scrapie represents a neurosis, characterised by symptoms of intense and progressive pruritus, progressive asthenia and locomotor incoordination. The disease is known to have been present in Britain for more than 200 years and although the conditions of its occurrence were well recognised, these presented very conflicting indications in respect of its nature. While there was a considerable volume of field evidence which indicated that scrapie was an infective disease, there were also features which seemed to suggest that it did not represent an infective process but resulted from an hereditary diathesis, or was produced by some hypothetical inanimate agent present in the pasture (Greig, 1940a). Several workers (Dammann, 1869, M'Fadyean, 1918, Bertrand, Carré and Lucam, 1937) attempted to produce the disease experimentally by inoculating sheep with material derived from typical cases of scrapie and also by keeping healthy sheep in prolonged immediate contact with affected animals. All these attempts proved negative.

During an extended study of the conditions of occurrence of scrapie in the field the writer had observed several instances which suggested the possibility that the disease could be transmitted through the medium of the pasture, and in November 1932 there was commenced an experiment in which a small group of scrapie sheep—usually three of four—and a group of 26 healthy ewes were alternately grazed at intervals of three to four days in a small, enclosed paddock, care being taken that during the process of interchange the two groups did not come into immediate contact with each other. The experiment was a prolonged one; indeed a period of approximately 3 years and 3 months elapsed before the first evidence of scrapie appeared in the contact ewes which were then 25 in number (February, 1936). Between then and May, 1938, when the experiment was concluded 9 other contact ewes developed scrapie. In 3 of the 10 cases the diagnosis was not precisely determined, but the remaining 7 were typical cases of the disease and it was concluded that the investigation afforded experimental evidence that scrapie is an infective disease, that the incubation period is prolonged and that the causal agent can be transmitted through the medium of pasture (Greig, 1940b).

At the time of their purchase 6 of the contact ewes were in-lamb, and during the period of the experiment all the contact ewes were mated in November 1933 and again in November 1934 to one of their tup lambs; the ewe-lambs, the progeny from these matings, were maintained in the contact group and the wether lambs were disposed of to the butcher.

Louping-ill vaccine as prepared by the Animal Diseases Research Association consists essentially of a 10 per cent. saline suspension of brain, spinal cord and spleen derived from sheep five days after their infection with virus inoculated intracerebrally: formalin to produce a concentration of 0·35 per cent. is added in order to inactivate the virus. The vaccine is applied by subcutaneous injection.

In June, 1937 there were received several complaints to the effect that sheep vaccinated with our louping-ill vaccine in 1935 were now developing scrapie. These were shortly followed by a considerable number of similar complaints from different parts of Scotland and in several instances the affected sheep were blackfaces, a breed in which scrapie very seldom occurs under natural conditions. It quickly became apparent that the louping-ill vaccine of 1935 had in some manner become contaminated with the causal agent of scrapie, and this received confirmation in the fact that the vaccine had been prepared in 3 batches and the cases of scrapie were confined to those sheep which had received "Batch No. 2" of the vaccine. No complaint was received in respect of the sheep vaccinated with Batches Nos. 1 and 3. Eighteen thousand sheep had been vaccinated with Batch No. 2 but a large number of these were ewes of 4 years and upwards at the time of vaccination and had therefore been disposed of by the autumn of 1937. The incidence showed marked variation in the different flocks and ranged from 1 per cent. to 26 per cent. with an average of about 10 per cent. The vaccine in question was prepared in November and December, 1934 and the great majority of the vaccinations had been carried out in March, 1935. The earliest cases of scrapie recorded occurred in November, 1936; at that time the vaccine was not suspected but the affected farm had been previously quite free from the disease and there is strong presumptive evidence that these cases resulted from the vaccination.

In view of these happenings we immediately investigated the sources of the 114 sheep from which "Batch No. 2" had been prepared. They were of the blackfaced breed and had been obtained from 3 flockmasters on whose farms scrapie had not been known to occur, but it was also found that there had been included in the batch the tissues of 8 Cheviot wethers (born April, 1934), the progeny of the contact ewes in the experiment above described; these, instead of having been disposed of to the butcher as was intended, had been included with the other sheep from which Batch No. 2 was prepared.

At the time of this occurrence (November-December, 1934) all the 8 lambs in question had appeared to be in perfect health and, indeed, as has been stated above, clinical signs of scrapie did not appear in their fellow contacts until February, 1936—*i.e.*, about 15 months later.

Thus from this unfortunate affair in which several thousand sheep had been accidentally inoculated with scrapie-infective

material, and maintained over a prolonged period, there was obtained evidence from which it appeared justifiable to draw the following inferences: —

(*a*) that the infective agent of scrapie is present in the brain, spinal cord and/or spleen;

(*b*) that it can remain infective in a concentration of formalin of 0·35 per cent. over a period of at least 3 months (the approximate period which elapsed between the preparation of the vaccine, November-December, 1934, and its application, March, 1935);

(*c*) that it can be transmitted by subcutaneous inoculation;

(*d*) that the incubation period is prolonged; it may extend to at least 1 year, 8 months (the approximate period between the application of the vaccine—March, 1935—and the earliest recorded cases of scrapie—November, 1936).

(*e*) that, apart from the incubation period proper, the infective agent can remain latent but potentially infective in the tissues of the living animal for an indefinite period during which it is capable of being activated so that it produces manifest disease.

During the course of the investigation designed to determine whether scrapie could be transmitted through the medium of the pasture, two independent workers, Cuillé and Chelle (1936, 1938[a]) using inocula derived from the cerebrum, medulla and spinal cord of several sheep in the later stages of scrapie, inoculated 17 healthy sheep by the intra-cerebral, epidural, intra-ocular, subcutaneous and intra-cutaneous routes. Of these 17 experimental sheep nine died from intercurrent affections, but of the remaining eight animals, two which received a preparation of spinal cord by intraocular inoculation developed symptoms of scrapie 14½ and 22 months after inoculation; one sheep which received an emulsion of medulla epidurally and one which received this inoculum subcutaneously developed scrapie after intervals of 1 year and of 19½ months respectively; one other sheep which received an intracerebral inoculation of spinal cord developed scrapie 11 months after inoculation, while the three remaining experimental sheep continued in apparent health. It would thus appear that out of 17 experimental animals the positive transmission of scrapie had been effected in five cases.

In a further series of experiments by Cuillé and Chelle (1938[b]) two healthy lambs each received, partly by intra-ocular and partly by subcutaneous inoculation, a filtered emulsion (Chamberland L.3) of scrapie spinal cord. Both these lambs developed typical symptoms of scrapie 16 months later.

The work of Cuillé and Chelle therefore furnished further experimental evidence that scrapie was an infective disease and it also showed that the disease could be transmitted artificially by inoculation, that the causal agent is capable of passing a compara-

tively fine porcelain filter and that it therefore probably represented a filtrable virus.

In view of all this evidence it became highly desirable to study the infective agent particularly with the object of determining its nature. The incidence both of the natural disease and that produced artificially presents marked variation and may be of a low order; it was therefore necessary to arrange for a large number of experimental sheep which preferably could be kept concurrently under experiment and which should if possible be uniform in type and therefore closely comparable among themselves.

Accordingly a flock consisting of approximately 780 sheep in regular age groups was purchased; the experimentation conducted on these has been summarily reported by Gordon (1946) and it is hoped will form the subject of a future paper.

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STUDIES IN SCRAPIE

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INTRODUCTION.

In continuation of investigations reported by Greig (1940) and Gordon (1946) experiments were commenced in November, 1945 with the object of determining whether the infective agent causing scrapie is a filterable virus and, if so, whether experimental serial passage in sheep would be possible as a means of maintaining the agent for further study. As all evidence so far obtained indicates that the causative agent is in fact filtrable and capable of being maintained indefinitely by serial inoculations in the natural host, it is desired, in view of the protracted nature of the investigation, to report the results of these experiments in this paper. Animals that remained in health for one year after receiving gradocol membrane filtrate as inoculum were not kept under further observation for it was felt that, as the average incubation period of scrapie in the experiment had proved to be approximately six months, the development of scrapie, if such occurred in those surviving one year, could be due to infection contracted from the pasture since this might have been contaminated earlier by their affected neighbours. This possibility was not entirely eliminated in the case of animals in experiment in the serial passage of virus but as the experiment progressed it appeared very improbable that the pasture in this instance had influenced the results to any significant extent.

METHODS.

Filtrability of the Infective Agent.

Materials and Techniques.

Pooled tissues consisting of portions of the cerebrum, cerebellum mid-brain, medulla and cervical cord taken from each of three naturally-occurring scrapie cases were triturated finely and then suspended in a concentration of 1 in 10 in broth-saline mixture (pH 7·3). The whole was centrifuged at approximately 2,500 r.p.m. for half an hour. The supernatant fluid was collected and a portion, without receiving further treatment, was retained as inoculum for one group of sheep while the remaining portion was filtered before being used for experiment in other groups.

Preparation of Filtrates.

The supernatant fluid was clarified by filtration through kieselguhr and then through a gradocol membrane of 1·00μ A.P.D. It

was divided into four portions each being filtered through a different gradocol membrane of 0·65μ A.P.D. Samples of the filtrates thus obtained were numbered 1, 2, 3 and 4 and retained separately as inocula for sheep. Portions of filtrates Nos. 1 and 3 were mixed and similarly with Nos. 2 and 4 and these were filtered using for each mixture a different gradocol membrane of 0·41μ A.P.D. The filtrates obtained were used as inocula for sheep. Unfiltered material and all filtrates were stored at 0° to 4°C. for about 18 hours before being injected into the test animals.

Sterility Tests.

Approximately 2 c.c. of each filtrate was seeded on plain agar and blood agar slopes and in broth as a test for viable bacteria. During a ten-day period of incubation none of the cultures showed any evidence of bacterial growth and the filtrates—six in all—were accepted as having been free from bacteria.

Test Animals.

Eighty-three Cheviot wedder lambs approximately seven months old from two farms where scrapie had not been known to occur were used in the experiment. The animals were divided into three groups and after inoculation were placed in separate fields which were not adjoining. The inoculum was administered to each animal by the intracerebral route in a dose of 1 c.c. on 27th November, 1945. The groups were as follows: —

Group 1 comprised 18 lambs which received unfiltered supernatant fluid.

Group 2 comprised 36 lambs that were subdivided into four lots which respectively received gradocol membrane (0·65μ A.P.D.) filtrate No. 1, 2, 3 and 4.

Group 3 comprised 29 lambs that were subdivided into two lots each of which received a gradocol membrane (0·41μ A.P.D.) filtrate.

EXPERIMENTAL RESULTS

Scrapie subsequently developed in each lot of Group 2 and Group 3 animals and because of this, individual animals referred to later are recorded for convenience as belonging to either Group 2 or Group 3.

Initial Transmission.

The results of the experiment are summarised in Table I which shows that four cases of scrapie developed in Group 1 animals, ten cases developed in Group 2 and seven in Group 3. Judging by the incidence of cases occurring in the respective groups, filtration with gradocol membranes of 0·65μ and 0·41μ A.P.D. had no significant effect on the infectivity of the suspension used as inoculum. The

experiment confirms the transmissibility of scrapie and proves the infectivity of filtrates which are bacteria-free. It does not, however, make possible any statement as to the approximate size of the

TABLE I

Group	*Number of sheep injected*	*Number of sheep developing scrapie*	*Incidence of scrapie per cent.*	*Incubation Periods*	*Course of disease i.e. until death or destroyed due to locomotor inco-ordination*
(1) Unfiltered suspension	18	4	22·2	Case 1. 5 months Case 2. 5·75 months Case 3. 6·0 months Case 4. 6·0 months (Average = 5·7 months)	2·0 weeks 5·0 weeks 12 weeks 13·5 weeks (Average = 8 weeks)
(2) Gradocol membrane (0·65μ A.P.D.) filtrate	36	10	27·7	Case 1. 5·75 months Case 2. 5·75 months Case 3. 6·0 months Case 4. 6·0 months Case 5. 6·0 months Case 6. 6·5 months Case 7. 6·5 months Case 8. 6·5 months Case 9. 8·0 months Case 10. 6·5 months (Average = 6·35 months)	4 weeks 5 weeks 4 weeks 4·5 weeks 4·5 weeks 7·0 weeks 8 weeks 10 weeks 4 weeks 15 weeks (Average = 6·5 weeks)
(3) Gradocol membrane (0·41μ A.P.D.) filtrate	29	7	24·1	Case 1. 5·3 months Case 2. 5·3 months Case 3. 5·6 months Case 4. 5·75 months Case 5. 5·75 months Case 6. 7·0 months Case 7. 9·75 months (Average = 6·32 months)	5·5 weeks 6 weeks 7 weeks 7 weeks 11 weeks 12 weeks 6 weeks (Average = 7·7 weeks)

causative virus since the gradocol membrane with the smaller pore size (0·41μ A.P.D.) was not a limiting or retaining membrane.

The symptoms exhibited by affected animals varied little from case to case and were typically those of scrapie of the "itch type". The appearance of several cases at about the same time, with each

affected animal showing the well-known symptoms throughout a course which did not vary much considering the insidious nature of the disease, is regarded as a noteworthy feature of the experiment. Evidence of the existence of scrapie was first suspected on 19th April, 1946 in one animal in Group 3. The diagnosis was confirmed on 7th May, 1946 when symptoms of itch were marked. These persisted and became more prominent during the following five weeks until 11th June, 1946 when marked locomotor disturbances also developed. The animal was unable to stand or walk without support from 12th June to 14th June, 1946 when it died. A second sheep in Group 3, though not suspected as suffering from scrapie so early as the above animal, was regarded as affected on 7th May, 1946. Again symptoms of itch predominated from that day until 11th June, 1946 when inco-ordination in gait also was noticed. On 12th June, 1946 the animal was unable to rise of its own accord but after being helped to regain its feet was able to walk or run several yards before stumbling and falling down. Appetite was not lost but as the animal was unable to stand on 18th June, 1946 it was destroyed.

In Group 1 the existence of scrapie was confirmed in one animal on 27th April, 1946. Symptoms of locomotor disturbance developed rapidly; they were present on 1st May, 1946. The animal walked or trotted in a swaying fashion and the head was very unsteady. These symptoms persisted during the following six days but frequently the animal was able to rise only with help. Up to this time it had been bright and the appetite was good. Symptoms of intense itch had been present throughout the course. When unable to stand the animal was destroyed on 10th May, 1946.

In Group 2 scrapie was first suspected in two animals on 17th May, 1946, both of which showed symptoms of itch. This became more intense and there was noticeable loss in bodily condition during the succeeding three to four weeks. In one, marked inco-ordination in gait and ataxia developed on 10th June, 1946. On 11th, 12th and 13th June, although this animal could stand only when supported, it was still bright and anxious to graze. It died on 14th June, 1946. In the other animal similar inco-ordination in gait developed; it was destroyed on the 18th June, 1946 when unable to rise or stand.

A description of each individual case is unnecessary on account of considerable similarity in the symptoms shown by affected animals. Reference to Table I, however, shows the length of the incubation period of the experimentally produced disease in each animal and the course, in weeks, before death occurred or before the animal was destroyed because of the development of locomotor inco-ordination and a final inability to walk or stand. Since scrapie is insidious in its onset it is difficult to determine accurately when pruritus first develops unless animals are handled every day. On that account the incubation periods in some cases were possibly

several days to about a week less than those recorded in the Table.

When animals became noticeably affected with scrapie they were separated from those of the group to which they belonged and placed together in a separate field. Here the animals when resting were seen to be constantly biting and nibbling at almost any accessible part of their bodies, and when disturbed they usually moved to the enclosing fences and engaged in persistent and vigorous rubbing of their necks, sides and quarters. Not infrequently, especially in the terminal stage of the disease, animals fell between the wires to the other side of the fence where they were unable to regain their feet.

In all animals, as the disease progressed there was noticeable loss in body condition, but the extreme degree of emaciation so often seen in naturally acquired scrapie did not occur. When locomotor inco-ordination had reached such a stage that animals could no longer walk or stand, several, when lying on their sides, were seen actively grazing the pasture within their reach.

Post Mortem Findings.

Apart from traumatic lesions of the skin and subcutis occasioned by the vigorous rubbing or nibbling, there were no findings worthy of comment in any animal.

The experimental disease like naturally acquired scrapie, is progressive. The animals in the above experiment, however, having been approximately only seven months old when the infective material was introduced into the central nervous system, developed scrapie when twelve to thirteen and one half months old —an age at which the naturally occurring disease has rarely or possibly never been observed. Out of 83 animals inoculated, 21 (approximately 25 per cent.) developed scrapie and the majority of these had died or were in the terminal stage of the disease by the time they were 14 to 15 months old.

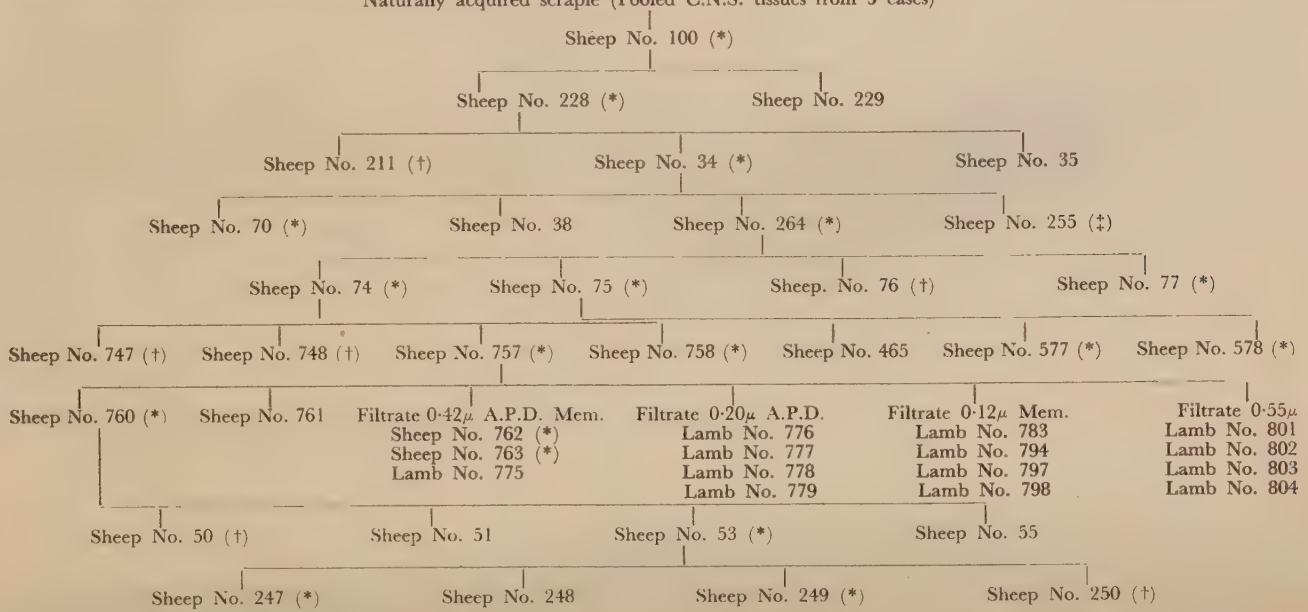
Passage of the Infective Agent Serially in Sheep.

As a means of maintaing the infective agent for further study, serial passage in sheep was begun using as inoculum pooled cerebrum, cerebellum, mid-brain, medulla oblongata and spinal cord, triturated and suspended in a concentration of 1 in 10 in broth-saline mixture. This suspension was centrifuged for one hour at 2,500 r.p.m. and the supernatant fluid was used as inoculum for sheep 228 and 229, each of which received a dose of 1 c.c. injected intracerebrally on the day the donor animal (Case 1, Group 1) was destroyed. The same system was adopted in subsequent passages with the exception that spinal ganglia were included in the pool of tissues, saline was used as diluent and the inoculum was administered to at least three sheep.

Passage of the agent serially proved to be possible and Table II shows the results of the series of intracerebral inoculations. Details

TABLE II

SERIAL PASSAGE OF SCRAPIE VIRUS IN SHEEP



C.N.S. = Central nervous system.
(*) = Animal developed scrapie.
(†) = Animal developed symptoms of itch which were transient but no locomotor inco-ordination or progressive emaciation.
(‡) = Died from debilitating disease without showing symptoms of itch.

Remaining animals maintained normal health.

TABLE III

SUMMARY OF SCRAPIE CASES RESULTING FROM SERIAL PASSAGE OF THE INEFECTIVE AGENT

Sheep No.	*Breed*	*Age when injected in months (approx.)*	*Serial passage*	*Incubation period in months*	*Course of disease (until animal was destroyed due to locomotor inco-ordination).*
100	Cheviot	7	First	5	2 weeks
228	Half-bred	1 year	Second	4	2 weeks
34	Half-bred	5 to 6	Third	5	4 weeks
70	Grey-face	1 year	Fourth	6½	4¼ months
264	Grey-face	1 year	,,	3¾	? Destroyed at 3½ weeks
74	Grey-face	16	Fifth	4	2¾ months
75	Grey-face	16	,,	4	2 weeks
77	Grey-face	16	,,	5¾	4½ months
577	Black-face	8	Sixth	17½	2 days*
578	Black-face	8	,,	6¾	9 days
757	Cheviot	1 year	,,	4½	1½ months
758	Cheviot	1 year	,,	5	4 months
760	Cheviot	16 or 17	Seventh	4	1 month
762	Cheviot	16 or 17	,,	6	3 months
763	Cheviot	16 or 17	,,	5½	1 month
53	Cheviot	10	Eighth	4½	? Destroyed at 3 weeks
247	Cheviot	15	Ninth	4½	? Destroyed at 16 days
249	Cheviot	15	,,	4½	3¼ months.

* Period of locomotor inco-ordination (see appendix).

of the individual sheep inoculated are given in the Appendix and a summary of scrapie cases which developed in the series is given in Table III.

Excluding Oxford—cross lambs which received "non-infective" filtrates through gradocol membranes in which the average pore diameter was less than 0·42μ, 17 cases of scrapie developed among 33 animals receiving infective material from the 2nd to the 9th passage. Of the remaining 16 animals, one (255) died from an undetermined cause, six showed transient symptoms of itch without the development of locomotor disturbance and nine maintained normal health. Over these serial passages (Table II) the development of scrapie has varied from a maximum of three cases out of four animals in the 5th passage to the minimum of one case out of four in the 8th passage.

Excluding Blackface sheep 577 used in the 6th passage, the incubation period of scrapie following intracerebral inoculation was found to be approximately five months and there was no obvious tendency for the period to change as a result of continued serial passage (Table III). Survival period after the first appearance of symptoms has varied from about two weeks in some animals to a maximum of four to four and one half months in others. Within the limits of six to seventeen months at the time of injection, no correlation could be established between the age of animal and the incubation period of the experimental disease or survival period of affected animals, but it may be worthy of record that of 14 animals admitted to the Institute as established clinical cases of naturally acquired scrapie during the past four years, seven survived more than five months whereas, of 42 experimental cases recorded in the present paper, none survived so long as five months. The difference in survival period of experimental and naturally acquired scrapie cases should not be accepted at present as an indication of enhancement in the virulence of the infective agent resulting from passage, for naturally acquired scrapie occurs nearly always in sheep aged two years and over and this fact may account for the relatively longer survival period in these animals. Moreover, no information has been obtained regarding survival period of experimental cases produced in aged sheep by intracerebral inoculation or in young sheep following inoculation of the passage strain by routes other than the intracerebral.

In those animals (211, 76, 747, 748, 50 and 250) which developed transient itch, scrapie was diagnosed, but because of the absence of progressive wasting in bodily condition, the previous diagnoses were subsequently doubted and no definite opinion was thereafter formed. This was on account of the present lack of a suitable serological test and the reluctance, at the time, to destroy these animals for sub-inoculation of other sheep while the hope remained that typical clinical scrapie might develop.

It will be noticed by reference to Tables II and III that serial

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

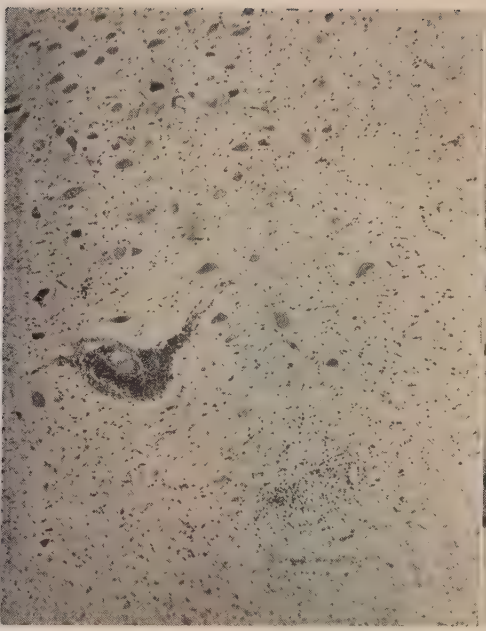
FIG. 1. Sheep No. 415. Appearance on 9th February, 1950—eight days after scrapie was diagnosed.

FIG. 2. Sheep No. 415. Appearance on 10th March, 1950. Fleece now showing evidence of hard rubbing.

FIG. 3. Sheep No. 415. Appearance on 18th March, 1950—two days before being destroyed. Animal was able to rise only with help and stand when supported.

FIG. 4. Sheep No. 403. Appearance on 29th March, 1950—19 days before animal died.

FIG. 5. Sheep No. 249. Affected with scrapie in ninth passage. Appearance on 6th February, 1950—22 days before death.



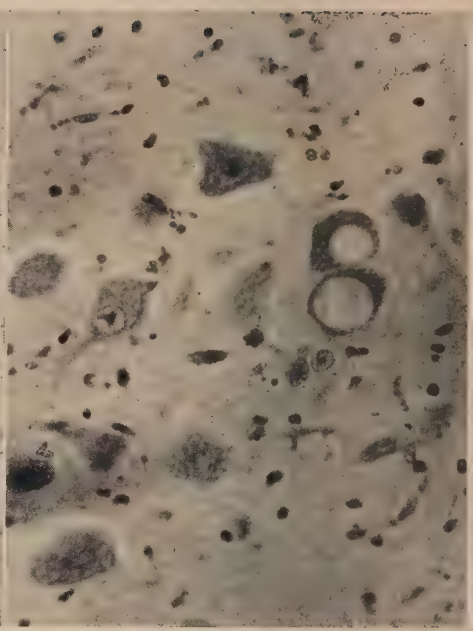


FIG. 6. FIG. 7.

FIG. 6. Sheep No. 53. Eighth passage scrapie. Medulla showing two vacuolated cells and diffuse and focal microglial reaction. H.E. ×80.

FIG. 7. Two vacuolated cells present in Fig. 6. H.E. ×640.

FIG. 8. FIG. 9.

FIG. 8. Sheep No. 264. Fourth passage scrapie. Cerebrum showing lymphocytic infiltration of the pia mater and neighbouring reaction in the plexiform and pyramidal cell layers of the cortex. H.E. ×320.

FIG. 9. Sheep No. 53. White matter of cerebellum showing vascular " cuffing " and diffuse and focal microglial reaction. H.E. ×80.

passage of the infective agent was accomplished by using as inocula the nerve tissues of cases where the course of the disease was short or, on occasion, where the affected animal was destroyed early in the course of the attack. The one exception to this rule concerned sheep 74 (5th passage) which was affected with scrapie for about 12 weeks before being destroyed for sub-inoculation tests. Whether it is important to use inoculum material as described is not known. The authors would record here their failure to transmit the infection to any of 40 sheep when using the central nervous tissues of two rams that had been affected with naturally acquired scrapie for long periods: in one the period was not less than six months and in the other, although the period of illness was unknown, death had occurred naturally in an advanced phase of the disease.

Viability of the Infective Agent in Dried Brain and Spinal Cord.

Portions of brain and spinal cord of sheep 228, 34, 264, 74, 757, 760 and 53, which respectively developed scrapie in the second to the eighth passage of the infective agent, were dried and also stored over C_aCl_2 *in vacuo* at 0° to 4° C. for the purpose of testing the viability of virus in the dried state. All seven dried-tissue samples were of material proved positive when inoculated in the fresh condition (Table II). The dried-tissue samples triturated individually and suspended in a concentration of 1 in 30 in saline, were spun at 2,500 r.p.m. for one hour and the supernatant fluids were used as inocula for 18 five-month old Cheviot sheep each of which received 1 c.c. intracerebrally on 13th September, 1949.

Since scrapie has appeared in a proportion of these animals it is accepted that the infective agent remains viable in the dried state for an undetermined period.

Scrapie developed in one sheep (412) of three which received dried brain and spinal cord of sheep 760 (storage period 225 days); one (415) of two which received dried brain and cord of sheep 53 (storage period 70 days); one (403) of two which received dried brain and cord of sheep 264 (storage period two years one month); and two (399 and 400) of three which received dried brain and cord of sheep 34 (storage period two years five and one half months). In all five, the symptoms which were marked were first seen in the animals in the order given after incubation periods of 3·75, 4·5, 4·75, 7·0 and 5·25 months respectively. Sheep 412 was destroyed when suffering from locomotor inco-ordination at the end of a disease course which lasted 22 days; sheep 415 (Figs. 1, 2 and 3) was destroyed at the end of a course of illness which lasted seven weeks; Sheep 403 (Fig. 4) died on 17th April, 1950 after a course of illness of ten weeks; sheep 399 survives at the present time (May, 1950) and sheep 400 was destroyed on 5th May, 1950 after a course of illness of about ten weeks.

Cerebro-spinal Fluid in Scrapie.

Samples of cerebro-spinal fluid were obtained from seven sheep

affected with naturally acquired scrapie, from 15 affected with scrapie produced experimentally and from 14 normal sheep. Several samples, up to a maximum of four, were taken at intervals from individual animals. The examinations which comprised cell counts, colloidal gold tests (Lange) and biochemical analyses revealed no feature by which these different groups of fluids could be distinguished one from another. Whether the infective agent is ever present in the cerebro-spinal fluid of affected animals is not known.

HISTOLOGY

Continued serial passage of the causal agent did not produce any apparent progressive changes in the intensity of the effects of the infection on the central nervous system. Throughout the series of cases the histological changes observed conformed to those of naturally occurring scrapie as described by Besnoit and Morel (1898), Stockman (1926), Bertrand, Carré and Lucam (1937), Brownlee (1940) and Holman and Pattison (1943).

Vacuolation of the nerve cells of the medulla (region just anterior to the calamus scriptorius) was present in all cases and although, in some, vacuolation was severe to the extent of four to six affected cells having been observed in a single microscope-field, a more searching examination was usually required to demonstrate vacuolation in the neurons of experimentally infected sheep than in those of naturally occurring scrapie cases.

No direct relationship between duration of illness and development or frequency of vacuolated cells could be established. Typically large, circular or oval vacuoles in the medulla cells, while never numerous, were found as easily in the few animals destroyed early in the course of the scrapie attack as in those left to suffer the effects of the disease over a period extending to three or four months. Figures 6 and 7 show vacuolation of two cells in the medulla of sheep 53 (eighth passage) which had been affected with scrapie for only three weeks when destroyed. Vacuoles were not found in the large nerve cells of the spinal cord in any animal in the series, but occassionally were present in the nerve cells of the spinal ganglia.

In the experimental disease, meningeal lesions, represented by mild infiltration of lymphocytes into the pia mater in the depths of the cerebral sulci, occurred without regular distribution. Sometimes, in association with such lesions, there was a microglial reaction resulting in diffuse and focal accumulations of cells in the neighbouring plexiform layer or even extending into the pyramidal cell layers of the cortex (Fig. 8). Reaction on the part of the microglial elements, however, might occur in any part of the brain or brain stem; where present it was never intense and usually was less marked in degree than that shown in either of the illustrations (Figs. 8 and 9).

Vascular lesions, consisting of a perivascular infiltration of lymphocytes, also had a wide distribution, but only a small proportion of the vessels was so affected. The number of congregating lymphocytes was never great, but on occasion a layer of three or four cells deep was observed which, with few exceptions, did not completely encircle the affected blood vessel. Nerve cells showing satellitosis were frequently observed.

The histological changes which are widespread in the brain and brain stem of experimental scrapie sheep indicate the existence of a subacute or chronic meningo-encephalitis.

DISCUSSION

The variations in incidence of experimental scrapie in the groups of sheep employed has been of considerable interest in the present investigation. Thus, while in one experiment only approximately 25 per cent. of the sheep developed scrapie as a result of inoculation with material obtained from naturally occurring cases, in serial transmission experiments, the strain of virus acquired was capable of inducing scrapie in small groups of sheep in proportions varying from 25 per cent. to 75 per cent. of the animals inoculated. In total, out of 33 animals employed in serial transmission of the agent, 17 (51·5 per cent.) developed scrapie and among the survivors six animals showed symptoms which, although proving transient, were referable to the existence of scrapie. If similarly affected sheep can subsequently be classed as representing atypical cases of the disease the authors consider the existence of an inapparent form of the affection as being a definite possibility. Such a hypothesis might account in part for the varying survival rates in the different groups of sheep which received proved virulent material by so favourable a route as the intracerebral.

While a long incubation period (seldom less than four months) was the rule, in experimental cases there were considerable variations in the effect of the virus on its host. Thus, in some animals inco-ordination or complete ataxia developed within ten days after symptoms of the disease first appeared, while in others, pronounced symptoms persisted for several months before ataxia and recumbency developed.

The routes by which natural infection takes place have not been determined and the means by which the virus subsequently reaches the central nervous system is at present unknown.

SUMMARY

Scrapie occurring in certain Scottish sheep stocks is an infective disease caused by a filtrable virus of undetermined size.

The disease is transmissable in series from sheep to sheep; in the present investigation nine successful passages have been made.

In some instances, the incubation period of the experimental disease, resulting from intracerebral inoculation, was approximately four months, but usually it was about five months.

The virus remains viable in the desiccated state at 0° to 4°C. for long periods; in each of two experiments virus survived in dried brain tissue for a period of over two years.

In addition to a slight and variable degree of vacuolation of the neurons in the medulla oblongata the histological changes observed in the central nervous system of sheep experimentally infected were indicative of the existence of subacute or chronic meningoencephalitis.

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APPENDIX

Histories of Sheep used in Experiment in Serial Passage of Scrapie Virus

First passage.—(Sheep 100). This was Case 1 of Group 1 animals in Filtrate Experiment (Table I). The animal—a Cheviot wedder —was approximately seven months old when inoculated on 27th November, 1945. Symptoms of intense itch, first noticed on 27th April, 1946, persisted throughout the course of the disease which lasted approximately two weeks. Locomotor inco-ordination which had developed on 1st May, 1946 became more pronounced on succeeding days until 10th May, 1946 when the animal was destroyed.

Second passage. (Sheep 228 and 229). These were Half-bred wedders approximately one year old when inoculated on 10th May, 1946. Sheep 228 showed symptoms of scrapie (itch) early in September, 1946 after an incubation period of about four months. Locomotor inco-ordination developed eight days after symptoms

were first observed and after a further five days the animal was destroyed (23rd September, 1946).

Sheep 229 remained in good health until 3rd August, 1948 when observation was discontinued.

Third passage. (Sheep 34 and 35). These were Half-bred wedders. (Sheep 211). This was a Scottish Blackface wedder. All were five or six months old when inoculated on 23rd September, 1946.

Sheep 34 commenced rubbing itself on 17th February, 1947 and scrapie was diagnosed on 27th February, 1947. During March rubbing was vigorous and persistent until the 24th and on this day locomotor inco-ordination developed, the animal being down and unable to rise without help. It was destroyed on 25th March, 1947.

Sheep 35 remained in normal health and observation was discontinued on 3rd August, 1948.

Sheep 211 was observed rubbing itself on many occasions during the period 25th September, 1947 to 18th March, 1948. There was no marked loss in body condition and as the animal subsequently appeared in good health, observation was discontinued on 3rd August, 1948.

Fourth passage. Sheep 38 was a Half-bred. Sheep 70, 264 and 255 were Grey-faces (Border Leicester x Blackface); all were one year old when inoculated on 25th March, 1947.

Sheep 38 remained in apparent health until 3rd August, 1948 when observation was discontinued.

Sheep 70 developed symptoms of itch towards the end of September, 1947. These symptoms persisted for several months and during the period there was considerable loss in body condition. Locomotor inco-ordination developed on 4th February, 1948 and the animal died from scrapie on 8th February, 1948.

In sheep 264 scrapie was first suspected on 18th July, 1947 as symptoms of itch had developed. Itch became more intense in degree and the animal was destroyed, affected with scrapie, on 11th August, 1947.

Sheep 255 after inoculation on 25th March, 1947, remained in apparent health and then lost body condition very considerably in the period between 4th February and 8th March, 1948. The animal was unable to stand on 8th March, 1948, and was destroyed on the following day when in the same condition. There had been no noticeable symptoms of itch at any time in the course of the illness. Unlike scrapie cases which show no macroscopic changes in the thoracic or abdominal organs, in sheep 255 the liver was very pale in colour and there was cirrhosis.

Fifth passage. Sheep 74, 75, 76 and 77 were Grey-faces. They were about 16 months old when inoculated on 11th August, 1947.

Sheep 74 and 75 were first noticed showing symptoms of itch on 10th December, 1947. In 74 the disease progressed in a manner typical of scrapie and during January and February, 1948, there was a marked itch and progressive emaciation with the develop-

ment of "cuddy trot gait." The animal was destroyed on 2nd March, 1948, in a condition of "advanced scrapie." In sheep 75 the disease progressed rapidly; itch persisted but on 21st December, 1947, locomotor inco-ordination was noticeable and on each of the following three days until 24th December, 1947 when it was destroyed the animal was able to stand and walk only after being helped to regain its feet.

Sheep 76 developed symptoms of itch early in February, 1948 and these persisted until the end of March and possibly until 7th April, 1948. There was little or no further evidence of disease and observation was discontinued a year later (13th April, 1949).

Sheep 77 first developed symptoms of itch on 4th February, 1948. On 18th March, 1948 symptoms of itch had become pronounced and there was noticeable loss of body condition. During April and May the disease progressed and besides the symptoms of intense itch, those of locomotor disturbances were displayed by the animal on being hurried. Between 5th June, 1948 and 15th June, 1948 the animal was usually able to stand and walk only after being helped to regain its feet. Emaciation became extreme and the animal was destroyed on 21st June, 1948 in the terminal stage of scrapie.

Sixth passage. Sheep 465, 577 and 578 were Blackfaces about eight months old. They were inoculated on 24th December, 1947. Sheep 747 and 748 were North Scotland Cheviots while 757 and 758 were South Scotland Cheviots, all approximately one year old when inoculated on 3rd March, 1948.

Sheep 465 remained in good health and observation was discontinued on 7th July, 1949.

Sheep 577 remained in good health until 8th June, 1949 when it was found down; it was able to remain on its feet, however, when steadied, but on the following day it was unable to do so even when supported. There was some evidence that the animal had been rubbing its buttocks and shoulders. Bodily condition was good. The animal was destroyed on 9th June, 1949 in the belief that the condition was scrapie though atypical.

Sheep 578 exhibited symptoms of scrapie (itch and inco-ordination in gait) during the period 15th July, 1948 to 22nd July, 1948. On several occasions it was found down and unable to rise without help. On 24th July, 1948 the animal was unable to stand and was destroyed.

Sheep 747 and 748 both developed symptoms of itch; in the former animal these became apparent on 5th October, 1948 and in the latter on 1st September, 1948. In both animals these symptoms remained evident until February, 1949; thereafter they were not again observed. Sheep 748 became noticeably poor in bodily condition but this may have been due to a cause other than scrapie. Both animals survived; observations on 747 were discontinued on 7th July, 1949 and on 748 on 20th December, 1949.

On 19th July, 1948 sheep 757 was observed vigorously rubbing and occasionally nibbling itself and scrapie was diagnosed. Itch remained very intense during August and there was much loss in body condition. Animal was destroyed on 1st September, 1948.

In sheep 758 the first symptoms of scrapie developed on 28th July, 1948. Thereafter the disease was slowly progressive and at the beginning of October, 1948 the condition was one of typical clinical scrapie. Symptoms of locomotor inco-ordination developed in November and the animal was destroyed as an advanced case of scrapie on 2nd December, 1948.

Seventh passage. Sheep 760, 761, 762 and 763 were South Scotland Cheviots, 16 or 17 months old when inoculated on 2nd September, 1948. Others (thirteen in all) were Oxford cross lambs three to four months old when inoculated with gradocol membrane filtrates of scrapie sheep brain on 2nd September, 1948. Two of these died from cause unconnected with scrapie, seven and nine months respectively after inoculation and the remaining eleven cross lambs showed no signs of illness throughout a period of observation until 7th July, 1949.

Of the Cheviots, three (760, 762 and 763) developed scrapie. In 760 the first evidence of this was noticed on 30th December, 1948. The disease progressed to a typical clinical form and on 31st January, 1949 the animal was destroyed. In sheep 762 scrapie first became evident on 27th February, 1949, when itch developed and became intense in March. In May the animal was attacked with convulsions on several occasions. Marked locomotor inco-ordination developed on 27th May, 1949 and the animal was destroyed on 30th May, 1949.

In sheep 763 the symptoms of itch were observed on 18th February, 1949 and ten days later they were well established. On 12th March, 1949 locomotor disturbances were noticed and on 14th March, 1949, when unable to stand, the animal was destroyed.

Eighth passage. In this passage Cheviot wedders, (50, 51, 53 and 55) were about ten months old when inoculated on 31st January, 1949. In sheep 53, the only member of the group which became affected with scrapie, symptoms were first observed on 14th June, 1949 and two weeks later itch was intense. The animal was destroyed on 5th July, 1949 before locomotor inco-ordination had developed.

In sheep 50, symptoms of itch which appeared to be mild in degree were noticed on 2nd November, 1949 and these have persisted without apparent change until the present time (May, 1950). There was no wasting in body condition. Whether the animal was affected with scrapie has remaind undetermined.

Ninth passage. Cheviot wedders (247, 248, 249 and 250), were approximately 15 months old when inoculated on 5th July, 1949. In two animals, 247 and 249, the existence of scrapie was suspected on 22nd November, 1949 but as none of the animals in the group

had been examined closely during the period of 20 days previous to this date, scrapie in either or both may have been present some days earlier. In sheep 247 itch increased in intensity and the animal was destroyed on 8th December, 1949 before locomotor disturbances had become evident. In sheep 249 itch also increased in intensity and during December, 1949 and January and February, 1950 the disease progressed in the manner characteristic of scrapie. The animal was destroyed on 28th February, 1950. Figure 5 in the text shows the appearance of this animal on 6th February, 1950, when symptoms of locomotor disturbances first became evident. In sheep 250 scrapie was suspected on 7th January, 1950, as symptoms of itch, though not well marked, were present. These symptoms persisted and the animal remained a suspected, though not a confirmed, case of scrapie. The remaining member of the group (248) maintained normal health up to the present time (May, 1950).

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STUDIES ON VITAMINS A AND C IN BOVINES.

VITAMIN C IN THE LIVER, KIDNEY AND PLASMA OF COWS, CALVES AND FOETUSES.

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INTRODUCTION

There have been many reports on the concentration of ascorbic acid in the serum of cattle (Sheahan, 1947), but there is no agreement on whether this concentration varies according to the season of the year.

Thus Wallis (1940) records no variation in the plasma levels during the year, while Sheahan (1947) states that her highest values were in the autumn and her lowest in the winter. If it is assumed that the milk values parallel the blood levels, then figures to support both views have been recorded. Trout and Gjessing (1939) record high blood values in the spring and low blood values in the winter, but the figures for milk given by Kon and Watson (1937), Guthrie (1939) and Wallis (1940) do not confirm this. As it is accepted that the ruminant is capable of synthesising vitamin C (Knight, Dutcher, Guerrant and Bechel, 1941; Wallis, 1942, 1943), the concentration of this substance in the blood and tissues in general would not be expected to vary according to the food supply and this was found to be the case by Brown, Van Landingham and Weakley (1939), Wallis (1940), and Bortree, Huffman and Duncan (1942).

These last authors recorded considerable variations for blood from individual animals when sampled at intervals of a few hours. Random sampling of relatively small numbers of animals would therefore be unlikely to produce a mean value without a considerable standard error, and it was therefore decided to estimate the ascorbic acid content of the livers of a large series of animals. This was considered likely to give a better indication of the actual vitamin level in the animal and to reveal any seasonal or dietary effects which might occur.

Relatively few estimations of the liver values have been recorded and these have varied. Thus Phillips and Stare (1934) give 25mg./100g. for the average of 3 cows, Weikenberg (1939) noted 20 to 25 mg./100g. in 43 cows, and Sinko (1936) reports that in 9 animals the mean value was 49.3mg./100g. of liver tissue.

In the present series of estimations the livers of 370 cows have been examined. In 367 of these the kidneys were also investigated while plasma samples were available from 197 cows. In addition 62 foetal livers and 144 calf livers were examined. Every sample came from an animal of pure Ayrshire stock bred in the locality. There were therefore no breed or climatic factors to affect the results.

EXPERIMENTAL PROCEDURE

The animals were slaughtered on the morning after they arrived at the abattoir; they had no access to food or water for at least 18 hours before death. Those from which it was intended to collect specimens were killed in a batch under the author's supervision. The number of each animal was noted and it was traced back through the market to its farm of origin. The owner was then contacted by telephone, letter or personal visit, and full details of the food fed to the animal were recorded. In addition, records were made of whether the animal was a heifer or a cow and whether it was pregnant. If it was pregnant the age of the foetus was estimated from its size and the liver removed for vitamin C estimation. All these data were obtained for most of the animals but in some, particularly those sent in by dealers, there were some omissions and where these animals are concerned the data in the tables are not complete.

Immediately following slaughter the specimens—blood, kidney and the spigella lobe of the liver—were taken to the laboratory and the ascorbic acid content estimated by the method of Harris and Olliver (1942). When estimating the vitamin C in calves the blood and kidney samples were omitted, only the liver being used. All estimations were in duplicate on duplicate samples, i.e., four estimations were made for each organ.

For liver and kidney 10g. of tissue were ground in a mortar for 2 minutes with 12·5 ml. trichloroacetic acid, the mixture filtered through muslin, diluted to 50 ml. with distilled water and the reducing activity estimated with dichloroindophenol under a daylight fluorescent lamp. The dye was prepared 24 hours before use and standardised against a known concentration of ascorbic acid during the course of the estimations. The blood samples were taken from the jugular vein into bottles containing oxalate and cyanide, 5 ml. of plasma being used for the estimations. Metaphosphoric acid was added as coagulant. To avoid possible personal variation each separate step of the preparation was invariably performed by the same person and all titrations were also carried out by a single worker. As it was possible that the liver varied in weight during the seasons, and therefore 1g. of liver in the summer might represent less of the total weight of the liver than 1g. in winter, 50 livers were weighed during the summer months and 50 during the winter. There was little difference between these weights, the average summer weight being 7·2kg. and the average winter weight 6·7kg. The work started in September, 1945 and concluded in June, 1947.

RESULTS

The whole series of estimations is summarised in Table 1 where the mean values, their standard errors and the maximum and minimum values are given. The mean amount of vitamin C

TABLE I.

VITAMIN C IN THE LIVERS, KIDNEYS AND PLASMA OF COWS

Month		LIVER					KIDNEY					PLASMA				
		No. of cows	*Mean value vit. C (mg./100g.)*	*S.E.*	*Max. (mg./100g.)*	*Min. (mg./100g.)*	*No. of cows*	*Mean value vit. C (mg./100g.)*	*S.E.*	*Max. (mg./100g.)*	*Min. (mg./100g.)*	*No. of cows*	*Mean value vit. C (mg./100ml.)*	*S.E.*	*Max. (mg./100ml.)*	*Min. (mg./100ml.)*
September	1945	24	25·2	0·87	35·6	17·0	24	8·0	0·34	10·9	5·5	—	—	—	—	—
October	1945	26	24·4	0·83	30·9	17·5	26	9·0	0·25	14·0	7·6	—	—	—	—	—
November	1945	28	23·0	0·81	31·1	17·7	28	8·2	0·28	12·3	5·2	—	—	—	—	—
December	1945	12	22·3	1·22	27·5	15·0	12	8·9	0·54	12·4	5·4	12	0·36	0·044	0·74	0·18
January	1946	23	25·0	0·88	30·2	20·3	23	10·0	0·64	19·9	7·5	23	0·33	0·023	0·54	0·15
February	1946	23	25·0	0·88	33·6	18·0	23	8·8	0·63	12·1	7·7	23	0·30	0·024	0·55	0·13
March	1946	18	22·3	1·01	28·3	11·5	18	8·4	0·42	12·1	6·1	18	0·27	0·022	0·46	0·16
April	1946	12	21·5	1·24	28·2	15·6	11	8·0	0·17	9·7	4·7	12	0·21	0·088	0·28	0·12
May	1946	17	22·9	1·04	27·5	16·9	18	8·2	0·33	12·5	6·3	18	0·24	0·016	0·37	0·17
June	1946	24	22·0	0·87	34·4	15·3	24	8·2	0·33	10·6	5·6	22	0·27	0·016	0·44	0·17
July	1946	15	21·2	1·10	26·0	16·0	15	7·4	0·28	9·0	5·4	15	0·23	0·018	0·28	0·14
September	1946	17	24·7	1·79	31·0	17·8	17	8·0	0·46	12·7	4·9	17	0·28	0·017	0·39	0·18
October	1946	29	23·1	0·79	29·5	16·9	29	7·8	0·22	10·1	5·0	29	0·22	0·008	0·38	0·16
November	1946	8	23·7	1·54	33·4	18·7	8	7·5	0·70	9·8	4·5	8	0·20	0·018	0·29	0·15
February	1947	11	20·1	1·29	27·5	12·8	8	6·7	0·58	8·9	4·1	—	—	—	—	—
March	1947	23	22·3	0·88	27·7	17·1	23	9·3	0·34	12·7	5·4	—	—	—	—	—
April	1947	20	25·6	0·96	28·5	15·8	20	9·3	0·33	11·3	6·8	—	—	—	—	—
May	1947	10	20·4	1·35	25·2	16·6	10	7·5	0·38	9·5	5·6	—	—	—	—	—
June	1947	18	20·7	0·84	26·2	13·6	18	8·2	0·40	12·2	5·6	—	—	—	—	—
July	1947	12	27·3	1·26	36·7	21·7	12	10·1	0·55	14·5	7·2	—	—	—	—	—
TOTAL...		370	23·2	0·23	36·7	11·5	367	8·5	0·61	19·9	4·1	197	0·26	0·0075	0·74	0·12

(or reducing substance) present in the liver was 23·2mg. per 100g. of liver substance, in the kidney 8·5mg. and in the plasma 0·26mg./100 ml. While the majority of the estimations were close to the

TABLE II

THE CONCENTRATION OF VITAMIN C RELATED TO TYPE OF FOOD

Food Group	*Mean Value of Vitamin C in mg./100g.*					
	Liver		*Kidney*		*Plasma*	
	No. of Animals	*Vit. C.*	*No. of Animals*	*Vit. C.*	*No. of Animals*	*Vit. C.*
A.	229	23·3	218	8·2	117	0·24
B.	89	23·6	86	9·3	49	0·29
C.	12	24·4	12	8·9	—	—
D.	3	24·6	3	9·0	—	—
E.	11	21·9	10	9·1	—	—

A. Animals with access to grass.
B. Animals without access to grass but with turnips in the diet.
C. Animals without access to grass but fed some grass meal.
D. Animals without access to grass, turnips, or grass meal but fed kale.
E. Animals fed only on hay, straw and cattle cake.

mean there were occasional large variations and the table therefore includes the maximum and minimum values.

The plasma results with a mean of 0·26mg./100 ml. are lower than those recorded by most authors though the range was con-

siderable. Thus Bortree *et al.* (1942) using 28 cows with a mean value of 0·44mg./100 ml. record maximum and minimum figures of 0·8 and 0·11 while Sheahan (1947) gives maximum and minimum figures of 0·92 and 0·14 for 250 cows. A closer range, 0·19 to 0·66, is recorded by Phillips, Lardy, Boyer and Werner (1941) for 154 cows. While individual differences may account for much of this variation (Bortree *et al.*, 1942) it is possible that breed or climatic conditions, especially extremes in temperature, may account for at least part of it. In this connection it is interesting to note that by far the highest figures are those given by Radeff (1941) with a mean of 1·32mg./100 ml. for 130 cattle. These are three times as great as those of most authors and five times as great as those obtained in the present work. Radeff was working under the same general conditions in S.E. Europe as Sinko (1936) whose values for the liver with a mean of 49·3mg./100g. are approximately twice those of other workers. Since greater amounts of ascorbic acid are present in the tissues of rats under cold environmental conditions (Dugal and Thérien, 1947; Thérien, Leblanc, Heroux and Dugal, 1949) it is possible that the mild equable littoral climate in south-west Scotland does not necessitate the maintenance of high levels of vitamin C in the animals, and that this is the cause of the low values in the peripheral circulation in the animals examined by us. Radeff and Sinko were both working under a continental type of climate.

In Table 2 the figures are arranged according to the type of food available to the animal for at least 14 days before death. All groups except "A" were fed hay and straw. The last three groups contained so few animals that the differences between the results for them and for the other groups were not statistically significant. There was no difference in the liver values in animals with access to grass or those fed turnips, but in the kidney and plasma the concentration was slightly higher in animals fed turnips as compared with those at grass (kidney 9·3 ± 0·20 and 8·2 ± 0·11; plasma 0·29 ± 0·017 and 0·24 ± 0·0065 respectively). These differences though small are significant (Table 3) and it might therefore be considered that the feed, while not affecting the actual vitamin reserves in the liver, does affect the level in the peripheral circulation and the kidneys. This might be so, since turnips are a rich source of ascorbic acid (McHenry and Graham, 1935). Nevertheless the bacterial flora which flourishes in the rumen during grass feeding is different in some aspects from that prevalent during winter feeding and this may have a bearing on the destruction of the vitamin in the rumen (Knight, Dutcher, Guerrant and Bechdel, 1941). While this possible food factor should not be excluded, a more probable explanation is that the period of turnip feeding corresponds to the coldest part of the year and hence to the period when the vitamin requirements are highest. (c.f. Thérien *et al.*, 1949).

TABLE III

ANALYSIS OF VARIANCE

Class of Data		*Components*	*Degrees Freedom*	*S.S.*	*Variance*	*F.*	*P.*
Kidney samples Grass compared with Turnips.	Total	304	303	1051·53	3·47		
	Groups	2	1	74·63	74·63	23·1	>0·0001
	Error	—	302	976·69	3·23		
Plasma samples Grass compared with turnips.	Total	166	165	1·89	0·011		
	Groups	2	1	0·09	0·086	7·8	>0·01
	Error	—	164	1·80	0·011		
Liver samples May and June compared with rest of year	Total	370	369	7577·04	20·53		
	Groups	2	1	213·87	213·87	10·69	>0·001
	Error	—	368	7363·17	20·0		
Liver samples Dam compared with foetus.	Total	100	99	4578·10	46·24		
	Groups	2	1	1423·36	1423·36	44·22	>0·0001
	Error	—	98	3154·74	32·19		
Liver samples Cows compared with calves.	Total	514	513	16208·9	31·59		
	Groups	2	1	3810·7	3810·72	157·4	>0·0001
	Error	—	512	12398·2	24·21		

The liver figures do not however follow the atmospheric temperature curve. In Table 1 they are arranged according to the month of the year and it will be seen that the lowest values are

TABLE IV

THE VITAMIN C CONCENTRATION IN LIVER, KIDNEY AND PLASMA RELATED TO PHYSIOLOGICAL CONDITION

Physiological Condition of Animal.	*No. of Animals.*	*Mean Vitamin C concentration in the*		
		Liver (mg./100g.)	*Kidney (mg./100g.)*	*Plasma* (mg./100 ml.)*
Adult Cow	229	23·0	8·4	0·28
Uncalved Heifer	29	24·1	9·3	0·27
Pregnant	128	23·4	8·7	0·28
Non-Pregnant	219	23·3	8·8	0·28

* Figures for plasma relate to 109 cows, 15 heifers, 35 pregnant and 102 non-pregnant animals only.

recorded in the late spring and early summer when temperatures are higher than for the winter months. A possible factor involving the temperature is that these months are the first the cows are

out at grass both by day and by night and it is therefore possible that there is an increased call on any reserves to maintain the peripheral level of ascorbic acid. The mean value of ascorbic acid in the liver for these two months was 21·6 ± 0·43 whereas for the rest of the year, taken as a whole, it was 23·8 ± 0·17. If these figures are analysed statistically the values in Table 3 are obtained. It will be seen that the difference is highly significant.

Another factor which might affect the level of the vitamin C is pregnancy. Bortree, *et al.*, (1942) stated that the plasma values did not vary in samples taken from 12 pregnant and 16 non-pregnant animals. Gosse (1936) however recorded higher figures for pregnant animals. Table 4 shows that in our series the mean concentration in 128 pregnant and 219 non-pregnant animals was identical in the liver and kidneys. Similar results were recorded for the plasma though fewer animals were used. Pregnancy does not therefore affect the concentration of ascorbic acid in these organs.

In addition to investigating the vitamin concentration in adult cows the opportunity was taken to ascertain if there was any relationship between the liver levels in the dam and foetus. No such relationship could be shown in a series of 50 in which the liver values in both dam and foetus were compared. High levels in the dam with low levels in the foetus and vice versa were frequently found. Nevertheless there was a consistently lower level in the foetus than in the dam. The mean concentration recorded in the livers of the dams was 23·8 ± 0·76 and in the foetuses 17·4 ± 0·92. When analysed statistically this difference was shown to be significant (Table 3). As the size of the foetus increased there was a slight but steady increase in the vitamin C concentration in the liver (Table 5).

No livers were available from foetuses about to be born or from calves at the moment of birth, but 24 calves were obtained before they were 12 hours old and a further 120 at different ages up to 7 days. The mean value of 32 ± 1·4mg./100g. recorded for calves less than 12 hours old was the highest mean of any series and parallels the results recorded for plasma by Lundquist and Phillips (1943) on calves up to 2 weeks old. Their results showed a slow drop during the first 14 days whereas ours for liver do not show any steady trend. The abrupt increase in ascorbic acid content of the liver at or immediately before birth may indicate the rapid mobilisation of the vitamin to deal with the abrupt environmental change occurring at this time. This abrupt increase is shown graphically in Fig. 1 which also relates the liver values of cows, calves and foetuses and shows clearly how the level in the foetal liver is less than that in the cow while the level in the calf is greater. If the vitamin content is closely linked with the homoiothermal system, these differing concentrations are simply explained by the heat loss from the surface areas.

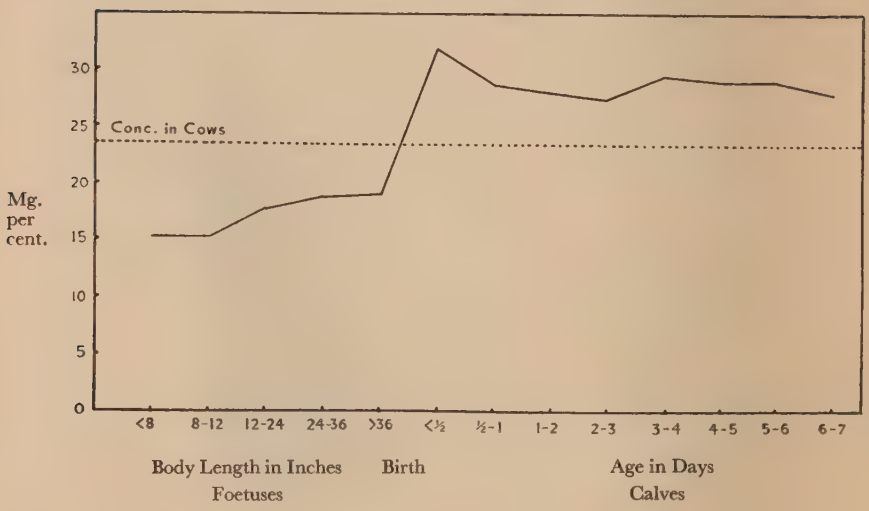
TABLE V

THE VITAMIN C CONTENT IN THE LIVERS OF FOETUSES AND CALVES

Foetal livers				*Calf livers*					
Size (in.)	*No.*	*Mean vit. C. content (mg./100g.)*	*S.E.*	*Age of Calf*	*No.*	*Mean vit. C. content (mg./100g.)*	*S.E.*	*Max.*	*Min.*
< 8	13	15·2	2·46	*Hrs.* < 12	24	32·0	1·4	41·9	20·0
8-12	22	15·2	0·43	12-24	20	28·7	1·4	42.9	20·7
12–24	15	17·9	2·6	*Days* 1–2	24	28·2	1·2	35·8	20·7
24–36	8	18·8	2·75	2–3	15	27·4	1·6	37·4	20·2
> 36	4	19·2	4·6	3–4	23	29·6	1·0	46·5	21·7
				4–5	15	29·1	1·7	43·0	18·2
				5–6	9	29·1	1·8	36·5	22·6
				6- 7	14	27·9	1·2	39·2	21·4
TOTAL	62	16·5	0·92	TOTAL	144	29·3	0·5	46·5	18·2

FIG. 1.

Concentration of Vitamin C in Livers of Foetuses and Calves.



SUMMARY

The mean values of vitamin C in the livers were as follows: 23·2 ± 0·23mg./100g. for 370 cows, 29·3 ± 0·50mg./100g. for 144 calves up to 7 days old and 17·4 ± 0·92mg./100g. for 62 foetuses. The mean value in the kidney in 367 cows was 8·5 ± 0·61mg./100g. and in the plasma of 197 cows it was 0·26 ± 0·0075mg./100 ml. The liver values were significantly less during May and June and the kidney and plasma levels significantly higher during the period of turnip feeding, i.e., during the colder months of December, January, February and March. The concentration in the livers of foetuses was significantly lower and in calves significantly higher than in cows. There was an abrupt increase in the concentration of vitamin C in the liver at the time of birth.

ACKNOWLEDGMENTS

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INFECTIOUS ENTEROHEPATITIS (BLACKHEAD) IN TURKEYS: A STUDY OF THE PROPHYLACTIC AND THERAPEUTIC VALUE OF CERTAIN COMPOUNDS

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INTRODUCTION

In a previous communication (Farmer and Stephenson, 1949), attention was drawn to the difficulty encountered in investigating the treatment of blackhead due to lack of adequate supplies of infected turkeys under controlled conditions, and a method was described whereby the disease could be produced at will. It was decided to use this technique to study the prophylactic and therapeutic properties of certain compounds and this article records the results of these investigations.

In considering the various chemotherapeutic substances described in the literature on blackhead, close examination of the results reveals a lack of reliable information in the records of many of the experiments. In many cases no reference is made to the number of poults used and much of the work consists of uncontrolled experiments or experiments in which the probable incidence of infection in the control and treated groups was quite unpredictable. The weakness of some of these trials was inherent in the method of inducing the disease, which usually depended on natural methods of infection. This is well illustrated by the work of Temperton, Dudley, Bythell and Parker (1947), in which neoarsphenamine was used prophylactically. No significant difference in the incidence of disease was demonstrated between the control and treated groups owing to an almost complete absence of blackhead in either group. The early experiments of Tyzzer (1923) on tryparsamide and neoarsphenamine included no control birds and the investigations on mapharsen by Bolin and Vardiman (1941) and McCulloch and Nicholson (1941) were also poorly controlled. Glover (1948) conducted an extensive trial of mapharsen therapy in naturally infected flocks but he concluded that in no instance had any of the sick birds benefited from the treatment. Similar criticisms apply to the trials of many of the non-arsenical preparations described. Emetine hydrochloride was used in only two cases by Tyzzer, Fabian and Foote (1921), and Wegeforth and Wegeforth (1921) gave ipecacuanha with some degree of success but their work was based on natural infection with no provision for control groups. Similar experiments with ipecacuanha using artificially infected birds showed a survival rate of 33 per cent. in the control group and treatment therefore remains unconfirmed; moreover, earlier work on this substance by Tyzzer and Fabian (1922) had failed to demonstrate any beneficial effect. Johnson (1934) tested colloidal iodine in a flock of 630 turkeys; the survival rate in the treated group was 93.1 per cent. and in the control group 89 per cent. survived.

Favourable reports have been made on acetarsol and mapharside (Blount, 1938; Hungerford, 1940) and on sulpharsphenamine (Hunger-

ford, 1940). It is difficult to assess the significance of most of the work reported in the literature owing to lack of numerical data or inadequate numbers of birds. However, the experiments conducted by DeVolt and Holst (1948) introduced a real advance in technique and have adequately demonstrated the prophylactic value of vioform (5-chloro-7-iodo-8-hydroxyquinoline) when incorporated in the mash at a concentration of 1 per cent. The treated groups showed a survival rate of 84.7 per cent. and in the untreated birds 3.6 per cent. survived. It is worthy of note that these workers used cultures as a means of inducing an artificial infection under well-controlled conditions.

Further work on vioform by Morehouse (1948) has confirmed its prophylactic value against artificial infection under controlled conditions produced by means of embryonated heterakis eggs, and the effective concentration has been shown to be 0.5 per cent. More recently, Waletsky, Brandt, Bliznick and Hughes (1949) and Brackett and Bliznick (1949), using a similar method of infection, have reported favourably on a preparation "Enheptin T" which was used therapeutically.

A review of this subject would not be complete without some mention being made of the use of phenothiazine in the control of blackhead. It has been postulated that the caecal worm, *Heterakis gallinae,* acts as an intermediate host for the causative organism, *Histomonas meleagridis,* and that the removal of the worm is therefore an effective measure for the prevention of blackhead infection. The evidence in support of this is, however, not conclusive and recent work by Wehr and Oliver (1946) indicates that whilst phenothiazine is an efficient anthelmintic, it has little effect on blackhead. A series of experiments by these workers failed to demonstrate any difference in the incidence of natural blackhead in birds treated with phenothiazine as compared with untreated birds.

Apart from the work of DeVolt and Holst on vioform, there seem to have been few attempts to conduct well-controlled investigations into the efficiency of many of the drugs at present employed for treating the disease, and the aim of the present work has been to provide some basis for the assessment of the value of the arsenical preparations now in use and also to investigate the possible effect of other non-arsenical substances. A list of the compounds tested is given in Table I.

METHODS

Each of the substances was passed through a screening test designed to give information as to the prophylactic and/or therapeutic possibilities of the drug. The procedure adopted for all substances was closely similar, viz., the treatment and infection of 12 four-week-old turkey poults, divided into 4 groups of three, as shown in Table II.

The method of infection was by passage from infected poults. Four turkeys infected with blackhead were killed on the 10th day after infection and the caeca removed, films from these being examined to confirm the absence of coccidia. The caeca were finely chopped and emulsified in 70 ml. Ringer's solution and the emul-

TABLE I

LIST OF SUBSTANCES TESTED AND TECHNICAL DETAILS OF INDIVIDUAL SCREENING TESTS

Exp. No.	*Chemical Constitution*	*LD/50 mgm/gm.*	*Dose given*	*Method of Dosage*	*Frequency*
1	Arsenious trioxide 1 per cent.	—	0·05 ml.	O	Daily
2	Neoarsphenamine	—	0·005 g.	I.M.	Every 3rd day
3	Sulpharsphenamine	—	0·005 g.	I.M.	Every 3rd day
4	Arsphenamine diglucoside 1·25 per cent.	—	0·4 ml.	I.M.	Every 3rd day
5	Acetarsol	—	0·25 g.	O	Daily
6	5-chloro-7-iodo-8-hydroxyquinoline (Vioform)	—	1% in food	O	Constantly
7	Antimony 5-chloro-7-iodo-8-hydroxyquinolinate	1·0	0·1 g.	O	Every 2nd day
8	Bismuth 5-chloro-7-iodo-8-hydroxyquinolinate	>14·0	1·5 g.	O	Daily
9	3:5:6:3^1:5^1:6^1-Hexachloro-2:2^1-dihydroxy-diphenylmethane	0·2	0·02 g.	O	Daily
10	Formaldehyde-sulphathiazole condensation product	10·0	1·0 g.	O	Daily
11	2-N^4 (α-N^1-Pyridiniumpropionyl) sulphanilamidothiazole bromide ...	>5·0	0·5 g.	O	Daily
12	α K-Tetra-*n*-amyldiaminodecane dihydrochloride	1·0	0·02 g.	O	Daily
13	Iodobismuthate of quinine	—	0·01 g.	I.M	Every 3rd day
14	Bismuth salt of ethylhydrogen cyclohexane 1:1 diacetate	—	0·01 g.	I.M.	Every 3rd day
15	Mepacrine hydrochloride	—	0·08 g.	O	Daily

O = Oral.
I.M. = Intramuscular.

TABLE II

SCREENING TEST FOR DRUGS

Group	*1st-14th day*	*15th day*	*15th-28th day*	*42nd day*
1	Drug given	All poults infected with blackhead	Drug given	All survivors killed and examined
2	Drug given		No drug given	
3	No drug given		Drug given	
4	No drug given		No drug given	

sion was filtered through a sieve to remove coarse particles. Each poult received an enema of 10 to 15 ml. of Ringer's solution immediately before injection and following this 2·5 ml. of emulsion were injected rectally into all poults on two occasions with an interval of two hours between injections. The emulsion was injected by means of a syringe fitted with a 6-inch length of rubber tubing of

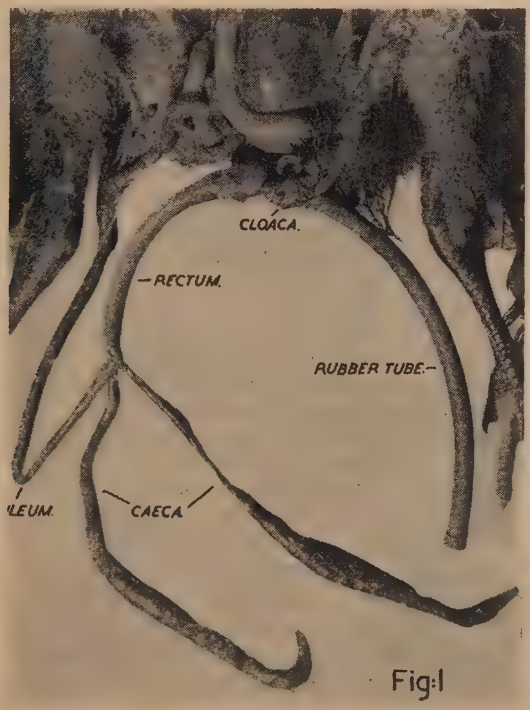

Fig:1

suitable bore. Four inches of tubing was passed into the rectum before injection and the location of the tubing in relation to the caecum is shown in Fig. 1.

This procedure was adhered to in all experiments, the only variable factors being the amount of drug given, the mode of administration and the frequency of dosage; these varied according to the nature of the substance under test and details are presented in Table I.

RESULTS

Preliminary Experiments

These were all in the nature of screening tests and the general procedure already described was adhered to. The number of each experiment corresponds to the index number of the drug being tested.

Arsenious trioxide, 1 per cent. (No. 1). The mode of administration was oral, the dose being 1 ml. of a 1 in 20 dilution of the concentrated solution. This amount was given daily in accordance with the directions issued with this preparation when used as a prophylactic. All poults died by the 18th day after infection and a diagnosis of blackhead was confirmed in all cases by post-mortem examination.

Neoarsphenamine (No. 2). This drug was injected intramuscularly at intervals of 3 days. The amount given was 0.005 g. dissolved in 0.5 ml. sterile distilled water. All poults had died of blackhead by the 18th day after infection.

Sulpharsphenamine (No. 3). The procedure for this experiment was identical to that for Experiment No. 2 except that sulpharsphenamine was used in place of neoarsphenamine. All poults died of blackhead by the 17th day after infection.

Arsphenamine diglucoside, 1.25 per cent. (No. 4). The general procedure was similar to that adopted in the two previous experiments, the dose being equivalent to 0.4 ml. per lb. body weight, which is comparable to 0·005 g. of the other arsenical preparations. The injection was made intramuscularly every 3rd day. All poults died of blackhead by the 13th day after infection.

Acetarsol (No. 5). This compound was available in the form of tablets (4 grain) which were given daily by the oral route. The results of this experiment were encouraging and details of the fates of the various groups were as follows: —

All poults in Group 1, i.e. receiving drug before and after infection, and in Group 2, i.e. receiving drug before infection only, survived and at post-mortem on the 42nd day showed no evidence of blackhead. Of those poults receiving the drug after infection only, i.e. Group 3, one poult died of blackhead on the 19th day after infection, but the remaining poults in that group survived and were quite healthy when examined post-mortem at the conclu-

sion of the experiment. All infected poults in the control group, i.e. Group 4, died of blackhead within 15 days of infection.

5-chloro-7-iodo-8-hydroxyquinoline (Vioform) (No. 6). This preparation was incorporated in the food at a concentration of 1 per cent. All poults in Group 1 survived and showed no evidence of blackhead at post-mortem examination on the 42nd day. In Group 2, one poult died of blackhead but the other poults remained free of the disease. All poults in Groups 3 and 4 died of blackhead.

The next six compounds had not previously been used in turkeys and, accordingly, toxicity tests in mice were carried out to determine their LD/50. As a general rule the dosage used in the subsequent experiments was one quarter of the LD/50, but full details of treatment and dosage for all substances tested are given in Table I. LD/50 figures are expressed in terms of mg/g. body weight in mice, whereas dosage rates are in g/lb. body weight in turkey poults.

Antimony-5-chloro-7-iodo-8-hydroxyquinolinate (No. 7). This drug was relatively insoluble and was prepared in the form of tablets, each containing 0.1 g. Toxicity tests indicated that it was toxic if given daily and the dose was therefore restricted to 0.1 g. every 2nd day. All poults in Groups 1, 2 and 4 died of blackhead as did one of the birds in Group 3. Of the two remaining birds in this group, one died of an intercurrent disease on the 3rd day after infection, but the other surived and showed only caecal lesions at post-mortem examination on the 42nd day.

Bismuth 5-chloro-7-iodo-8-hydroxyquinolinate (No. 8). This substance was of low toxicity and it was possible to administer as much as 1.5 g. daily without ill effect. This amount was given in the form of 6 tablets, each equivalent to 0.25 g. All poults in Groups 1 and 2 died of blackhead. In Groups 3 and 4 two poults died of blackhead in each group, but the survivors from both groups showed no evidence of blackhead at post-mortem examination at the conclusion of the experiment.

3 : 5 : 6 : 3′ : 5′ : 6′-Hexachloro-2 : 2′-dihydroxy-diphenylmethane (No. 9). The toxicity of this drug was relatively high and the dosage was therefore restricted to daily administration of one tablet containing 0.02 g. All poults in Groups 2, 3 and 4 died of blackhead by the 14th day after infection. Two poults in Group 1 also died of blackhead, but one bird survived and showed no sign of infection at post-mortem examination.

Formaldehyde-sulphathiazole condensation product (No. 10). The low toxicity of this substance enabled a dose of 1·0 g. per day to be given in the form of 4 tablets each containing 0·25 g. Birds in all groups died of blackhead with the exception of one in Group 2 which survived and showed no evidence of infection when killed for post-mortem examination at the conclusion of the experiment.

2-N^4 (α-N′-Pyridiniumpropionyl) sulphanilamidothiazole bromide (No. 11). This substance was also administered in the form of

tablets (0·25 g). Two of these were given daily, the total dosage being, therefore, 0.5 g. All the poults in each group died of blackhead.

αK-Tetra-n-amyldiaminodecane dihydrochloride No. 12). This substance was toxic at one quarter of the LD/50 and the dose was, therefore, reduced to 0·02 g. which was administered daily by mouth in the form of 2 ml. of a 1 per cent. solution. All poults in Groups 2 and 3 died of blackhead. In Group 1, two infected poults died of blackhead but the remaining poult survived until the end of the experiment. When killed for post-mortem examination, blackhead lesions were found in the caeca but the liver was apparently normal. Only two poults were used for Group 4 of which one died of blackhead and the other showed only caecal lesions at post-mortem examination at the conclusion of the experiment.

Iodobismuthate of quinine (No. 13). This substance was given in the form of a 10 per cent. emulsion, 0·1 ml. of which was injected intramuscularly at three-day intervals. All poults in Groups 1, 2 and 3 died of blackhead except one in Group 3 which died from caecal rupture on the day of infection. In Group 4 only one poult died of blackhead, the two other poults showing no evidence of blackhead at subsequent post-mortem examination. It was not thought that the failure of these two controls to contract blackhead invalidated the results obtained with the other groups in this experiment.

Bismuth salt of ethylhydrogen cyclohexane 1 : 1 *diacetate* (No. 14). The dosage and mode of administration was similar to that used in the previous experiment. All poults in Groups 2, 3 and 4 and two poults in Group 1 died of blackhead. The remaining poults in this group survived and showed no evidence of blackhead at the end of the experiment.

Mepacrine hydrochloride (No. 15). This substance was soluble and treatment was given daily in the form of 4 ml. of a 2 per cent. solution (0·08 g.). All poults in Groups 1 and 4 and two poults in Group 3 died of blackhead. The survivor in Group 3 remained healthy as did also one poult in Group 2. Of the remaining two poults in Group 2, one died five days after the commencement of the experiment from an unknown cause and the other died of blackhead.

The results of the screening tests are shown in Table III and are depicted in terms of deaths from blackhead in the four groups. The table shows that only acetarsol and vioform had any significant effect and it was obvious that further work would have to be concentrated on these two substances. Before continuing with these experiments it was decided, in view of the claims made for certain compounds in the treatment of blackhead, that the screening test with one of the arsenical preparations should be repeated, and accordingly an experiment on the lines depicted in Table II was conducted with arsphenamine diglucoside at an increased dosage

rate of 1 ml. in place of the original 0·4 ml. The treatment showed some effect in Group 1 in which two poults survived, the remaining poult dying of blackhead. In Group 2, two poults died of blackhead and one survived, but in Groups 3 and 4 all poults died of blackhead soon after infection. These results showed that at the increased dosage, arsphenamine diglucoside had a prophylactic effect which was, however, not nearly so marked as that of acetarsol.

Two more sets of experiments were now carried out to investigate the value of vioform and acetarsol and these are considered separately.

Value of Vioform (*5-chloro-7-iodo-8-hydroxy-quinoline*)

The second series of experiments consisted of two investigations, (1) to determine the minimum effective prophylactic dose and (2) to ascertain the therapeutic possibilities of the drug if used at a higher dosage rate.

Minimum effective prophylactic dose, (No. 16). Sixteen poults were divided into 4 groups of four. Group 1 received 1 per cent. vioform in the mash, Groups 2 and 3 received 0·5 per cent. and 0·1 per cent. vioform respectively and Group 4 was fed ordinary mash. Poults in all groups were infected after 15 days treatment. The birds in Groups 1 and 2 all survived and showed no evidence of blackhead at post-mortem examination on the 42nd day. Three poults in Group 3 died of blackhead but the survivor remained healthy. All poults in Group 4 died of blackhead. Thus vioform given prophylactically in the mash was effective at a concentration of 0·5 per cent.

Therapeutic Action, (No. 17). Fifteen poults were divided into 5 groups of three, and all of them were infected on the same day. Administration of the drug was delayed until after infection and Groups 1, 2, 3 and 4 then received 5 per cent. vioform in the mash commencing respectively on the 2nd, 4th, 6th and 8th days after infection. Group 5 received ordinary mash as did the other groups prior to commencement of treatment. Infected poults in all groups had died of blackhead by the 14th day after infection. It was also noted that the food was unpalatable due to the high concentration of vioform present.

Value of Acetarsol

The third series of experiments was devised in an attempt to find the answers to the following questions: (1) Has acetarsol a therapeutic as well as prophylactic action? (2) What is the minimum effective dose? (3) Would an increased dose have a therapeutic effect at an advanced stage of the disease, i.e. on the appearance of clinical symptoms? (4) What is the best method of administering the drug?

Therapeutic action, (No. 18). Experiment No. 5 had already indi-

TABLE III

DEATHS FROM BLACKHEAD IN THE FIRST SERIES OF EXPERIMENTS

Name of Substance	*Group 1*	*Group 2*	*Group 3*	*Group 4*
Arsenious trioxide 1 per cent.	3/3	3/3	3/3	3/3
Neoarsphenamine	3/3	2/3*	3/3	2/3
Sulpharsphenamine	3/3	3/3	3/3	3/3
Arsphenamine diglucoside 1·25 per cent.	3/3	2/3	3/3	3/3
Acetarsol	0/3	0/3	1/3	3/3
5-chloro-7-iodo-8-hydroxyquinoline (Vioform)	0/3	1/3	3/3	3/3
Antimony 5-chloro-7-iodo-8-hydroxyquinolinate	3/3	3/3	1/3*†	3/3
Bismuth 5-chloro-7-iodo-8-hydroxyquinolinate	3/3	3/3	2/3	2/3
3 : 5 : 6 : 3^1 : 5^1 : 6^1 -Hexachloro-2 : 2^1 dihydroxy-diphenylmethane	2/3	3/3	3/3	3/3
Formaldehyde-sulphathiazole condensation product	3/3	2/3	3/3	3/3
2-N^4 (α N^1-Pyridiniumpropionyl) sulphanilamidothiazole bromide	3/3	3/3	3/3	3/3
α K-Tetra-*n*-amyldiaminodecane dihydrochloride	2/3†	3/3	3/3	1/2†
Iodobismuthate of quinine	3/3	3/3	2/3*	1/3
Bismuth salt of ethylhydrogen cyclohexane 1 : 1 diacetate	2/3	3/3	3/3	3/3
Mepacrine hydrochloride	3/3	1/2*	2/3	3/3

Numerator = No. of deaths from blackhead.
Denominator = No. of poults infected.
* = One bird died soon after infection from intercurrent disease.
† = One other bird survived but showed caecal lesions at post mortem examination.

cated the value of acetarsol as a prophylactic, and the object of this experiment was to determine the therapeutic value of the drug when administered at various stages after the onset of the disease. An increased dosage was given to give full scope to any therapeutic effect which the drug might possess and, accordingly, the daily dosage was increased to five tablets per poult, i.e. 20 grains (1·25 g. approximately).

Fifteen poults were divided into 5 groups of three. The treatment of Group 1 commenced one day after infection and that of Groups 2, 3 and 4 on the 3rd, 5th and 7th days after, respectively. Group 5 received no treatment and all groups were infected on the same day. All the poults died of blackhead by the 14th day after infection, thus establishing that acetarsol was of no therapeutic value.

In view of this finding it was decided to determine the optimum conditions for using acetarsol as a prophylactic, and three more experiments were carried out.

Administration in Mash, (No. 19). In this experiment the drug was administered in the mash in powdered form in three different concentrations. Sixteen poults were divided into 4 groups of four, and these were fed mash containing 0·25 per cent., 0·05 per cent,, and 0·01 per cent. respectively, the fourth group receiving ordinary mash only. The general plan of this experiment was different from that depicted in Table II as Groups 1, 2 and 3 received treated mash before and after infection. The percentages in the mash were determined on the assumption that the average daily consumption of mash is about 80 g. per poult, and it was already known that 4 grains (approximately 0·25 g.) was an effective daily prophylactic dose. Thus Group 1 would probably ingest more than the effective dose and Groups 2 and 3 proportionately less.

The results of this experiment were clear cut. All poults in Groups 1, 2 and 3 survived and showed no evidence of blackhead on the 42nd day at post-mortem examination, whereas all four poults in Group 4 died of blackhead within 14 days of infection.

Difficulty was experienced in distributing the powdered acetarsol evenly throughout the mash and it was deemed advisable to investigate the possibilities of a soluble form which would enable the minimum effective dose to be determined more accurately and which could be used in field work by incorporation in the drinking water.

Administration of Acetarsol in water (No. 20). A solution of acetarsol was prepared by taking 5 g. of powder and adding to it the equivalent amount of $N1/N_aOH$ (18 ml.) required to prepare the sodium salt. This solution was then made up to 100 ml. with distilled water and constituted a 5 per cent. solution, further dilutions being prepared from this stock.

Fifteen poults were divided into 5 groups of three, 4 of the groups being treated and the fifth acting as a control. Treatment

was maintained for 28 days, all poults being infected on the 15th day. Group 1 received a daily dose of 5 ml. of a 5 per cent. solution (0·25 g.), Group 2 received 1 ml. of a 5 per cent. solution (0·05 g.), Group 3 received 5 ml. of a 0·2 per cent. solution (0·01 g.) and Group 4 received 1 ml. of a 0·2 per cent. solution (0·002 g.). Group 5 received no drug.

The results of this experiment were as follows; all poults in Groups 1, 2 and 3 survived and showed no evidence of blackhead at post-mortem examination on the 42nd day, whilst all poults in Groups 4 and 5 died of blackhead by the 15th day after infection. From these results the sodium salt of acetarsol appeared to be prophylactically effective in solution and the minimum effective dose was in the region of 0.01 g. daily.

Persistence of Arsenic in the Tissues. In view of the possibility that arsenic was present in the carcases of the survivors, samples of liver, kidney and breast muscle were submitted to analytical examination, the results of which are shown in Table IV. These poults were killed 14 days after cessation of treatment, and in all cases the arsenic content was within the prescribed limit of 1·4 parts per million.

Table IV

Persistence of Arsenic in the Tissues Fourteen Days after Cessation of Treatment

Group	*Daily Dose of Arsenic*	*Total Amount of arsenic given*	*Arsenic content p.p.m.*		
			Liver	*Kidney*	*Muscle*
1	0·25 g.	7·0 g.	<0·5	<0·5	<0·5
2	0·05 g.	1·4 g.	<0·5	1·0	<0·5
3	0·01 g.	0·28 g.	<0·5	<0·5	<0·5

The next step in the evolution of the soluble form of acetarsol was clearly to establish its value if incorporated in the drinking water, and to find what concentration would be necessary. The following experiment was therefore carried out.

Administration of Acetarsol in the drinking water (No. 21). From the results of the previous experiment it was established that 0·01 g. given daily as a prophylactic was an effective dose and from observations on the habits of turkey poults it was deduced that the probable consumption of drinking water for a month-old poult was of the order of 50 to 100 ml. per day. Accordingly, it was decided to give acetarsol in solution in three concentrations, namely 0·25 per cent., 0·05 per cent. and 0·01 per cent., on the assumption that the lowest concentration (0.01 per cent.) would be equivalent to the minimum effective dose if 100 ml. per day were consumed.

Thirteen poults were therefore divided into 4 groups of three, with an additional uninfected poult in Group 1 as control on the toxicity of the drug. The first three groups were given drinking water containing the above concentrations and the fourth group received ordinary water, treatment being continued for 10 days prior to infection and for 14 days after infection. The amount of water, and consequently the amount of arsenic consumed by each group was measured daily and details are presented in Table V.

TABLE V

ADMINISTRATION OF ACETARSOL IN THE DRINKING WATER. AVERAGE CONSUMPTION PER POULT.

	Day	*Group 1 0·25 per cent.*	*Group 2 0·05 per cent.*	*Group 3 0·01 per cent.*	*Group 4 No drug*
Before infection	1	87 ml	80 ml	82 ml	70 ml
	2	94 ,,	75 ,,	102 ,,	85 ,,
	3	100 ,,	102 ,,	102 ,,	90 ,,
	4	132 ,,	111 ,,	98 ,,	95 ,,
	5	119 ,,	113 ,,	124 ,,	87 ,,
	6	142 ,,	123 ,,	115 ,,	112 ,,
	7	95 ,,	88 ,,	65 ,,	75 ,,
	8	131 ,,	123 ,,	115 ,,	103 ,,
	9	131 ,,	131 ,,	118 ,,	110 ,,
	10	125 ,,	138 ,,	115 ,,	103 ,,
	11	147 ,,	133 ,,	124 ,,	114 ,,
	Total vol/poult	1,303 ,,	1,217 ,,	1,160 ,,	1,044 ,,
	Total arsenic/poult	3·27 g.	0·61 g.	0·12 g.	—
After infection	12	166 ml	142 ml	142 ml	117 ml
	13	170 ,,	157 ,,	150 ,,	107 ,,
	14	131 ,,	122 ,,	103 ,,	115 ,,
	15	150 ,,	159 ,,	120 ,,	55 ,,
	16	162 ,,	122 ,,	105 ,,	30 ,,
	17	145 ,,	153 ,,	127 ,,	132 ,,
	18	175 ,,	152 ,,	147 ,,	95 ,,
	19	179 ,,	169 ,,	160 ,,	120 ,,
	20	200 ,,	180 ,,	163 ,,	132 ,,
	21	160 ,,	140 ,,	130 ,,	120 ,,
	22	185 ,,	169 ,,	162 ,,	110 ,,
	23	192 ,,	177 ,,	157 ,,	—
	24	200 ,,	183 ,,	182 ,,	—
	25	186 ,,	185 ,,	165 ,,	—
	Total vol/poult	2,401 ,,	2,210 ,,	2,013 ,,	—
	Total arsenic/poult	6·0 g.	1·1 g.	0·2 g.	—
	Total amount of arsenic consumed per poult ...	9·27 g.	1·71 g.	0·32 g.	

All poults in Group 4 died of blackhead by the 13th day after infection, but all birds in the treated groups survived. Individual survivors within each group were killed at intervals of 2 days, 10 days and 18 days after cessation of treatment, and after post-mortem

examination, which revealed no evidence of blackhead infection, the arsenical content of liver, kidney and breast muscle was determined in all cases. The results of these analyses are shown in Table VI.

TABLE VI

ADMINISTRATION OF ACETARSOL IN THE DRINKING WATER. PERSISTANCE OF ARSENIC IN THE TISSUES.

Interval between last dose and arsenic estimation	*Tissue*	*Average amount of arsenic consumed per poult during experiment in :*		
		Group 1 9·27 *g.*	*Group 2* 1·71 *g.*	*Group 3* 0·32 *g.*
		arsenic content in parts per million.		
2 days...	Liver	2·0	<0·5	<0·5
	Kidney	<0·5	<0·5	<0·5
	Muscle	<0·5	<0·5	<0·5
10 days...	Liver	1·2	<1·0	1·2
	Kidney	1·5	<1·0	1·5
	Muscle	<1·0	<1·0	<1·0
18 days...	Liver	1·0	<1·0	<1·0
	Kidney	1·5	1·8	1·2
	Muscle	<1·0	<1·0	<1·0

The amount of drinking water consumed by the birds in the various groups showed a tendency towards a higher intake in the case of those groups receiving more arsenic and this tendency was constant from group to group throughout the experiment. It was not clear, however, whether this was due to increased palatibility of the water in direct relation to its arsenic content or conversely to the desire to imbibe more fluid in order to dilute the arsenic already taken.

The results of the arsenic estimations showed that most of the values were within the prescribed limit of 1·4 parts per million and that levels for muscle in every case were less than 1·0 part per million. In Group 3 one sample of kidney gave a value of 1.5 parts per million 10 days after cessation of treatment, but further samples from this group tested after an interval of 18 days all gave values below 1·4 parts per milion. All other levels above the permitted limit were from Groups 1 and 2, but taking into consideration the high dosage given to these groups, the amounts quoted are not excessive, the greatest value being 2·0 parts per million from the liver of a poult killed only 2 days after cessation of treatment.

Combined Results of Acetarsol Administration

The collected results of prophylactic treatment up to this stage using various concentrations of acetarsol are depicted in Table VII which shows that out of a total of 39 birds treated, only 3 died of blackhead and these were receiving an obviously sub-prophylactic dose (0·002 g.). All untreated birds, which numbered 13 in all, died of blackhead.

TABLE VII

PROPHYLACTIC RESULTS OBTAINED WITH VARIOUS CONCENTRATIONS OF ACETARSOL

Approximate Daily Dose of Acetarsol	*Number of Poults Infected*	*Number of Deaths from Blackhead*
0·25 g.	16	0
0·05 g.	10	0
0·01 g.	10	0
0·002 g.	3	3
—	13	13

Confirmation of the Prophylactic Effect of 0·01 per cent. Acetarsol in the Drinking Water, (No. 22).

Having shown the prophylactic value of acetarsol as a daily dose of 0·01 g., it was necessary to confirm its prophylactic effect when incorporated in the drinking water at a concentration of 0·01 per cent. A large-scale trial was obviously desirable but due to lack of inoculum it was decided to conduct five small-scale experiments. Each of these consisted in having 12 poults, all of which were infected on the same day, 6 receiving acetarsol in the drinking water and six having ordinary water. Treatment was given for 14 days before infection and continued for a further two weeks after infection. In four of the experiments the acetarsol was given in the same form as for Experiment No. 21 but in the fifth experiment a soluble tablet of sodium acetarsol was used. The results of these tests are shown in Table VIII.

Of the 30 birds treated, only one contracted blackhead and this group therefore shows a death rate of 3.3 per cent. The incidence of infection in the untreated groups was not so high as in previous experiments and in Test A this was due to an excessively dilute inoculum. The total death rate for the treated birds was 76·9 per cent.

TABLE VIII

PROPHYLACTIC RESULTS OBTAINED WITH 0·01 PER CENT. ACETARSOL IN THE DRINKING WATER.

Test	*No. of poults infected*	*No. of poults treated*	*No. of deaths in treated birds*	*No. of deaths in control birds*
A	12	6	0	3
B	12	6	1	5
C	12	6	0	4
D	12	6	0	5
E	12	6	0	5
Total ...	60	30	1	22

DISCUSSION

Vioform has been widely used in amoebiasis for many years (Anderson and Koch, 1931 etc.) and its value as a prophylactic in blackhead has already been described by DeVolt and Holst (1948). The results presented in this report confirm previous work on blackhead by these authors and establish also that the drug is still effective at a lower concentration (0·5 per cent.) than that reported, which confirms the work of Morehouse (1948). Unfortunately, it has not been possible to demonstrate any therapeutic effect and it seems unlikely that this substance has any future in the treatment of blackhead as it is much less effective weight for weight than acetarsol, the ratio of effective prophylactic dose being 50: 1.

The experiments described in this paper illustrate the advantages of oral administration of acetarsol as compared with intramuscular injection of other arsenicals. It is probable that the advantages do not lie entirely in the chemical nature of the drug and that some of its efficacy is due to the regular daily administration which ensures that an effective concentration of the drug is present in the alimentary tract. It is clear from Experiments No. 5 and No. 18 that unless acetarsol is present in the intestine at the time of infection a prophylactic effect would be unlikely, and it is therefore not surprising to find that the other arsenical preparations injected intramuscularly at 3-day intervals fail to produce the same results. It is possible, of course, that daily oral administration of these substances might give results comparable with that of acetarsol, but it was not thought worth while to pursue this matter further as any such treatment on a large-scale would be uneconomic. In one experiment in the first series (Experiment No. 5) the dosage

rate of acetarsol was considerably higher than that of the other arsenical preparations, namely 0·25 g. daily as compared with 0·005 g. every third day, but subsequent experiments have, however, shown that acetarsol is effective at a dose of 0·01 g.

The evolution of a soluble compound in the form of the sodium salt has conferred further advantages on acetarsol as a prophylactic and that it can be given in the drinking water in a wide range of concentrations without apparent ill effect is demonstrated in Experiment No. 21. No signs of toxicity were observed in any of the poults treated, and the final arsenic content of the carcase does not preclude the possibility of it being eaten with perfect safety. In any case it may be assumed that any arsenic present would be in the relatively non-toxic form of acetarsol, and it is difficult to see why routine daily dosage should raise any problem in this connection.

The prophylactic effect of this drug, when given in the drinking water at a concentration of 0·01 per cent., is amply confirmed in Experiment No. 22 in which only one poult died of blackhead out of a total of 30 treated birds. The untreated poults in this experiment showed a death rate of 76.9 per cent. and the completed figures, when subjected to the chi square test gave a value of 33·6, which under the conditions of the experiment is equivalent to a value of $P = <0{\cdot}001$.

In conclusion, it must be emphasised that all the experiments described have been conducted with an artificially induced disease which is relatively more severe than the naturally acquired condition. Final conclusions on the efficacy of these preparations against the natural disease must, therefore, await trials on a larger scale in the field under normal conditions of poultry husbandry.

SUMMARY

Experiments are described in which various preparations used for the treatment of blackhead have been subjected to trial using an artificial method of infecting turkey poults.

Three of the substances tested have shown prophylactic properties viz., (1) Acetarsol in the drinking water in the form of the sodium salt at a concentration of 0·01 per cent.; (2) Vioform (5-chloro-7-iodo-8-hydroxyquinoline) incorporated in the food at a concentration of 0·5 per cent.; (3) Arsphenamine diglucoside at a dose of 0·0125 g./lb. injected intramuscularly at intervals of three days.

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Since the completion of this work an article has been published by Sautter *et al.* (1950). *Amer. J. vet. Res.* **81,** 120, in which attention is drawn to the use of acetarsol (Stovarsol) for the treatment of blackhead.

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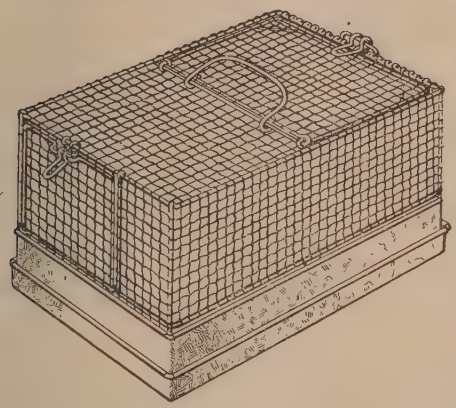

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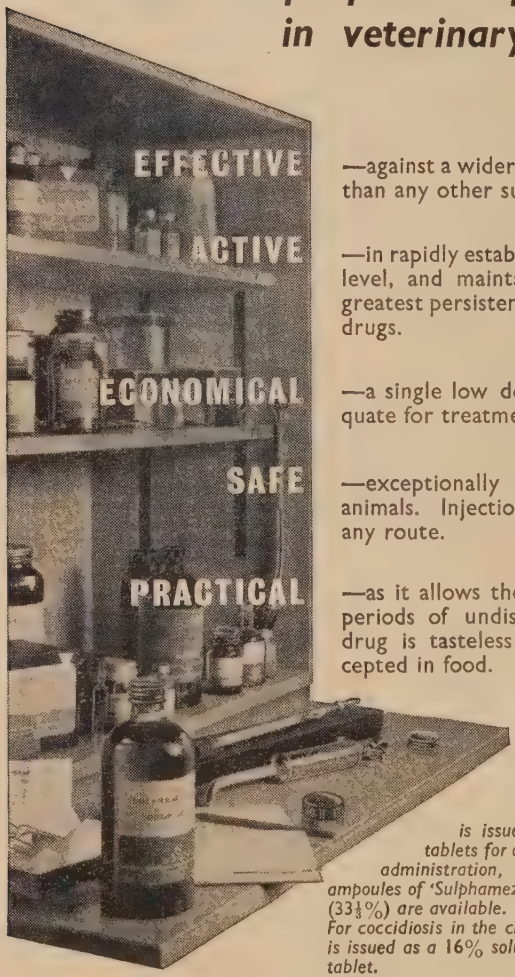

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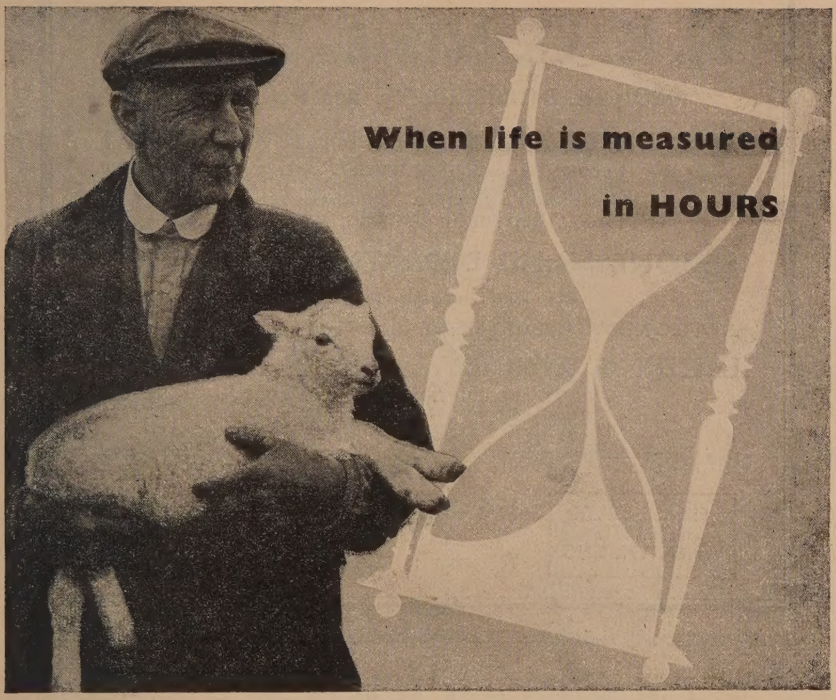

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